Use of peptide microarrays for mapping viral B cell epitopes

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Abbreviations

4608_RPL	random peptide library contained 4608 15-mere peptides
3D	three-dimensional
aa	amino acids
Ad5	adenovirus serotype 5
ADCC	antibody-dependent cellular cytotoxicity
AGL	antigenic loop of HBsAg
BB	blocking buffer
BCIP	5-Bromo-4-chloro3indolylphosphate toluidine salt
BSA	bovine serum albumin
C	constant domain of the immunoglobulin
CBS	citrate buffered saline
CDC	complement dependent cytotoxicity
CDR	complementary-determining region
CDS	color developing solution
CMV	cytomegalovirus
CST	conventional serological techniques
DMSO	dimethyl sulphoxide
DPZ	German Primate Center
e.g.	*exempli graciā* (for example)
E1	HIV vaccination experiment number 1
E2	HIV vaccination experiment number 2
E3	HIV vaccination experiment number 3
ELISA	enzyme-linked immunosorbent assay
EVA	European vaccine against AIDS
Fab	fragment antigen binding
Fc	fragment crystallizable
FcR	Fc receptors
Frs	framework regions
H	heavy chain of the immunoglobulin
HBcAg	hepatits B core antigen

HBeAg	hepatitis B e antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
HIVenv	HIV envelope
HPV	human papiloma virus
HSV	herpes simplex virus
HZI	Helmholtz Centre for Infection Research
i.e.	*id est* (that is; in other words)
ID	Intradermally
Ig	Immunoglobulin
IM	Intramuscularly
inf-Pa	HBV-infected patients
IV	Intravenously
L	light chain of the immunoglobulin
LMS	HBV-subviral particles bearing the 3 envelope proteins L-, M- and SHBsAg
mAb	monoclonal antibody
MBS	membrane blocking solution
MHBsAg	middle hepatitis B surface antigen
min	Minutes
MTT	thiazolyl blue tetrazolin bromide
MVA	modified vaccinia Ankara
MVAgpenv	modified vaccinia Ankara expressing HIV-1 envelop
NIBSC	National Institute for Biological Standards and Control, UK
PCC	peptide-cellulose conjugate
S/N	signal-to-noise ratio
SC^2	spotting compound – support conjugates
SCIV	single-cycle immunodeficiency virus vaccine
sec	seconds
SHBsAg	HBV small surface antigen
SHIV	simian-human immunodeficiency virus
SIV	simian immunodeficiency virus

SM	skimmed milk
T-TBS	Tween-Tris buffer solution
TBS	Tris buffer solution
TFA	trifluoroacetic acid
UMG	University Medical Center Göttingen
V	variable domain of the immunoglobulin
WMB	Whatman protein microarray blocking buffer

Amino Acids

A / Ala	Alanine
C / Cys	Cysteine
D / Asp	Aspartic Acid
E / Glu	Glutamic Acid
F / Phe	Phenylalanine
G / Gly	Glycine
H / His	Histidine
I / Ile	Isoleucine
K / Lys	Lysine
L / Leu	Leucine
M / Met	Methionine
N / Asn	Asparagine
P / Pro	Proline
Q / Gln	Glutamine
R / Arg	Arginine
S / Ser	Serine
T / Thr	Threonine
V / Val	Valine
W /Trp	Tryptophan
Y / Tyr	Tyrosine

1 Introduction

1.1 The immune responses

The environment contains an enormous range of pathogens and toxins that attack mammalian bodies. In response to these, mammals have developed their own defense, the immune system. The immune system eliminates these pathogens and toxins and thereby protects the organism. The immune response is organized in two stages. The first line of defense is the innate immunity, which is considered as the first line of defense against invading pathogens. It reacts rapidly after invasion of pathogens by recognizing molecular patterns shared by many microbes but not present on mammalian cells. The second line of defense is the adaptive immunity. This system recognizes individual pathogens through a large repertoire of specific receptors. Moreover, the adaptive immune system has a memory, which guarantees faster and stronger responses to reinvading pathogens (figure 1) (Abbas, Lichtman et al. 2010; Bonilla and Oettgen 2010; Turvey and Broide 2010).

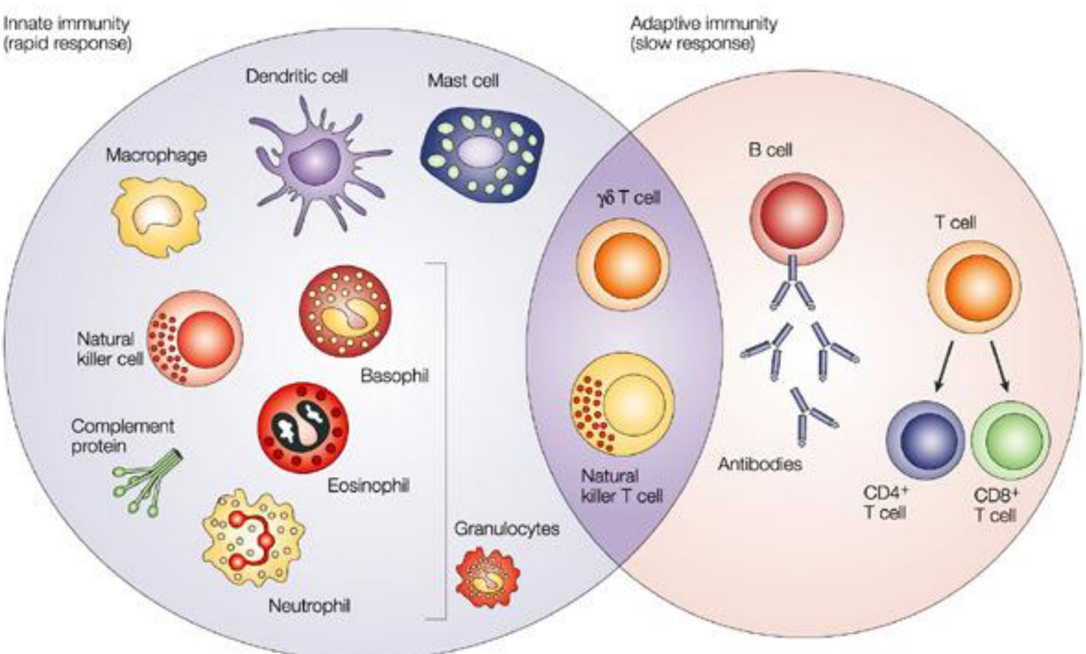


Figure 1: Major components of the innate and adaptive immunity (Dranoff 2004).

The adaptive immunity comprises two classes of cells. T -lymphocytes recognize intracellular microbes and function to destroy these or the infected cells. B -lymphocytes upon contact with the pathogen differentiate into plasma cells producing specific antibodies (Abbas, Lichtman et al. 2010).

1.2 Structure of the immunoglobulin antibody

As illustrated in figure 2, **i**mmuno**g**lobulin (Ig) is a heterodimeric protein composed of 2 **h**eavy (H) and 2 **l**ight (L) chains. The L chain can either be of a *K* or a λ type. H and L chains both contain an NH2-terminal variable (V) domain and COOH-terminal constant (C) domain. Each V domain consists of two sandwiched β-pleated sheets kept together by a disulfide bridge between two conserved cysteine residues. Each V or C domain consists of approximately 110 to 130 amino acids, comprising a molecular mass of 12,000 to 13,000 kd. Both Ig L chains contain only 1 C domain (light blue in figure 2), whereas immunoglobulin H chains contain either 3 or 4 such domains (dark blue in figure 2). H chains with 3 C domains include a spacer hinge region (black arrow in figure 2) between the first (C_H1) and second (C_H2) domain. This hinge gives the Ig the flexibility to bind to two determinants with variable distances. (Schroeder and Cavacini 2010).

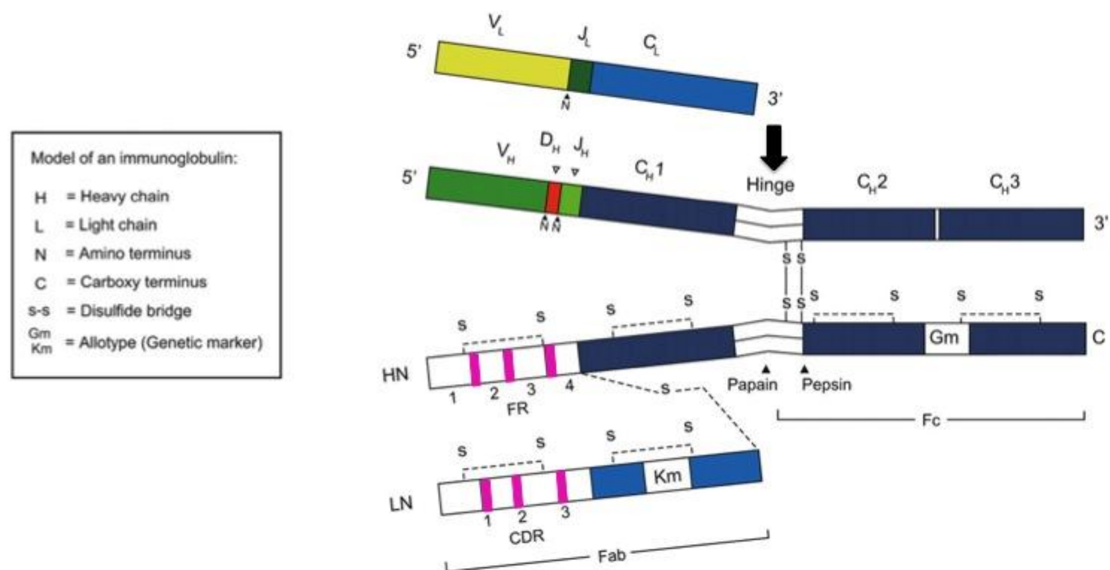


Figure 2: Two-dimensional model of an IgG molecule. For more details, see the text. Modified from (Schroeder and Cavacini 2010).

Pepsin digests Ig into two identical **F**ragment **A**ntigen **B**inding (Fab) arms, which still allow for bivalent binding. The Fab fragment consists of the entire L chain as well as the V and C_H1 domain of the H chain. The other fragment of the pepain is one **F**ragment **C**rystallizable (Fc) stem. The COOH-terminal part of the Fc fragment contains a site binding to **Fc r**eceptors (FcR) on effector cells. The antibody-bound pathogen can then be eliminated more effectively. (Smith, Nelson et al. 2004; Burton and Wilson 2007).

Within the V region of both L and H chains, there are three hypervariable regions, each 10 amino acids long. They are also called **c**omplementary-**d**etermining **r**egions (CDRs) because these sequences form a surface complementary to a 3D structure called the antigen (the CDRs are violet in figure 2). An example of antigen-antibody H chain binding is shown in figure 3 where the CDRs of a monoclonal antibody bind to **h**uman **i**mmunodeficiency **v**irus type **1** (HIV-1) gp120. The CDRs are interspaced with 4 regions of conserved sequence termed **f**ramework **r**egions (FRs) (the white area between the violet areas in figure 2) (Schroeder and Cavacini 2010).

A plasma cell can secrete one of the five Ig isotypes IgM, IgD, IgG, IgA and IgE, which differ in the Fc portion. In humans, IgA and IgG isotypes can be subtyped into IgA1 and IgA2, or IgG1, IgG2, IgG3, and IgG4 respectively. Each C domain of the H chain of a given isotope or subtypes has the same amino acid sequences. The C domains are designated by letters of the Greek alphabet corresponding to the Ig isotype. Thus IgA1 contains α1 H, IgA2 α2, IgD δ, IgE ε, IgG1 γ1, IgG2 γ2, IgG3 γ3, IgG4 γ4, and IgM μ. The IgG, IgA, and IgD have three C_H domains, while IgM and IgE have four (Abbas, Lichtman et al. 2010). The constant Fc region of the Ig isotypes or subtypes allow for the production of specific antibodies by immunizing heterologous species. Such antibodies are used as a second antibody in the analysis of humoral immune responses (e.g. with goat anti mouse IgG, the goat is the species immunized with mouse IgG).

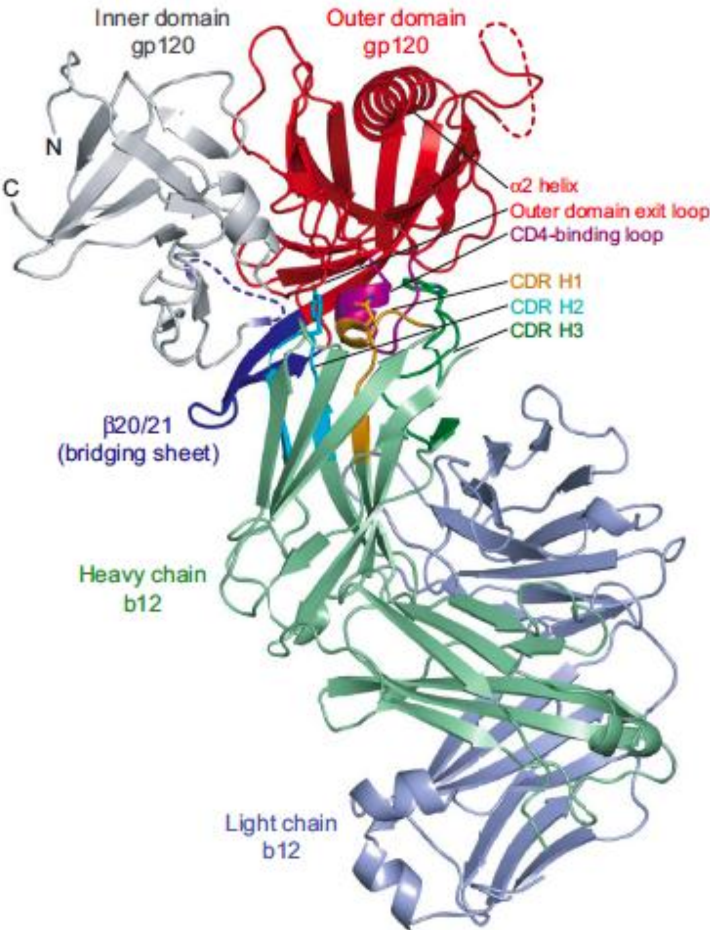


Figure 3: Interaction between a monoclonal antibody, b12 and HIV-1 gp120 core. The gp120 inner domain is grey, and the outer domain is red, except for the CD4-binding loop, which is purple. The strands and associated loops, which in the CD4-bound conformation correspond to the bridging sheet, are blue. The b12 light chain is blue–grey and the b12 heavy chain is green, with associated CDRs of the heavy chain highlighted in orange (H1), cyan (H2) and dark green (H3). The heavy-chain dominance of the binding interaction is apparent, with the nearest light-chain approach separated by ≈10A ° from gp120. Heavy-chain-only interactions are rare (Zhou, Xu et al. 2007).

1.3 Antigen-antibody interaction

The molecules capable of stimulating B -lymphocytes to produce antibodies are called immunogens (e.g. **h**epatitis **B** **v**irus (HBV)), while an antigen is any compound that binds to an antibody or T cell receptor (e.g. **h**epatitis **B** **s**urface **a**nti**g**en (HBsAg)). Usually antigens are much larger than the paratopes, the site of Ig binding to the antigen. Therefore, the antibody binds only to a portion of the antigen, which is called the epitope (Abbas, Lichtman et al. 2010; Schroeder and Cavacini 2010). The antigen may have several epitopes but the individual antibody binds only to one epitope.

Affinity is the strength of binding between an individual antibody and an epitope. A serum from an immunized person has antibodies of different affinities to the same antigen, depending on the amino acids sequences of the CDRs. The antibody is specific for the epitope. It can distinguish between minor differences in the chemical structure of an epitope (Chaplin 2010). However, some antibodies may react with a different but structurally related epitope or with a mimotope. The amino acid sequence of a mimotope is completely different from the related epitope but has the same chemical properties (Slootstra, Puijk et al. 1997; Meloen, Puijk et al. 2000).

1.4 Antiviral mechanisms of the antibody

Neutralization of the free virus particle is the first direct effect of the antibody, which takes place either extracellularly or intracellularly. Antibodies neutralize extracellularly through different mechanisms. (1) Antibodies block the virus ligand-cellular receptor thereby preventing viral attachment to the cell (Kolatkar, Bella et al. 1999). (2) Antibodies bind to the virus fusion protein inhibiting viral penetration into the cell (Mclain, Porta et al. 1995). (3) Antibodies inhibit virus release from the infected cell (Vanderplasschen, Hollinshead et al. 1997). Finally, antibodies can also block virus uncoating within the cell and thereby inhibit the virus nucleic acid release (Virgin, Mann et al. 1994).

Indirect effects of the antibody are mediated through its Fc part, which binds to FcR on the effector cell. This can activate several antiviral mechanisms. (1) Activation of the complement pathway leads to deposition of complement on the viral surface, neutralizing viral activity. (2) Activation of virus-bound complement can lead directly to virolysis (Avirutnan, Mehlhop et al. 2008). Finally, the Fc-mediated effector system can lyse virus-infected cells through either **a**ntibody-**d**ependent **c**ellular **c**ytotoxicity (ADCC) or **c**omplement-**d**ependent **c**ytotoxicity (CDC) (Burton 2002).

The neutralizing power of the antibody depends mainly on the recognized epitope. Discrimination between neutralizing and non-neutralizing epitopes is important for vaccine production and viral diagnostics.

1.5 Virus evasion of an antibody response

The antibody responses against a virus can stop it from spreading through the body,

can eliminate infectious particles and prevent reinfection. However, viruses can counteract neutralizing antibodies through evolutionary changes.

The influenza virus is a typical example for a virus evading the immune response through two distinct mechanisms. (1) An antigenic drift is due to point mutations in the hemagglutinin, and neuraminidase gens generate viral variants escaping antiviral neutralization. (2) An antigenic shift is caused by genetic reassortment of viral gene segment coding for the surface polypeptides hemagglutinin and/or neuraminidase. Thus, antibodies produced against the previously infecting virus cannot protect the host against infection with this new virus (Scholtissek 1995; Hilleman 2002; Vossen, Westerhout et al. 2002).

The antibody repertoire of the infected host exerts a high selective pressure on the infecting virus population. This pressure selects for changes in the neutralizing epitopes. Such changes can occur due to the low-fidelity of the RNA-dependent RNA polymerases. This escape mechanism is used by e.g. **h**epatitis **C** **v**irus (HCV). (Alcami and Koszinowski 2000; Diamond 2003). Other viruses (e.g. HIV) evade the antiviral complement activities by encoding homologues of complement regulatory proteins or incorporate host factors blocking complement activation into their lipid envelope, such as CD46 (Burton 2002; Diamond 2003). Others (e.g. cytomegalovirus and coronavirus) encode Fc receptors inhibiting the Fc-dependent immune responses (Alcami and Koszinowski 2000; Tortorella, Gewurz et al. 2000).

1.6 Immune memory

Specific antibodies appear after the first exposure to an antigen. This primary response is slow. First IgM with low affinity are produced after one week of exposure to the antigen. After two weeks, IgG with higher affinity start to appear and an immune memory is established. Activated B cell clones differentiate into antibody secreting cell and long-lived memory cells. After subsequent exposure to the same antigen, a high-affinity IgG is produced within the first week of secondary response. (Abbas, Lichtman et al. 2010; Bonilla and Oettgen 2010).

1.7 Vaccination

Vaccination is a unique way to employ the immune memory. Vaccines are an important tool to combat and even eradicate infectious diseases. For example, in the first three-quarters of the 20th century, up to 300 million people died from smallpox. In contrast, due to a massive vaccination program, no one has died from the disease since 1978 (Burton 2002).

Vaccinations mimic natural infections without causing disease. As antigens, toxins, surface structures of the pathogens or their killed or attenuated forms are applied (Burton and Parren 2000). Vaccines induce antibody responses. Similar to the antibodies found after natural infection, vaccine-induce antibodies neutralize pathogens e.g. HBV, measles and influenza viruses (Ada 2001; Plotkin 2008). The titer of antibodies induced by vaccination is an indicator of protection. HBV-vaccinated individuals with 10 IU/l of specific antibodies are considered to be protected and those with lower levels or no antibody titers should be revaccinated (Mahy and Ter Meulen 2007).

1.8 Serology

Serology is defined as the immunochemical analysis of antibodies or antigens present in sera or other body fluids.

1.9 Standard serological methods for the detection of antibodies

Antibodies are usually detected and quantified by binding to a fixed quantity of one known antigen (e.g. whole or recombinant virus or synthetic peptides) attached to a solid phase (e.g. plastic microtiter plate). Test serum or dilutions thereof are added to antigen-containing wells and incubated at room temperature. Unbound antibodies are removed by washing. A second species-specific antibody linked to an indicator is allowed to bind. The indicator can either be a radioisotope, an enzyme or fluorophore. This assay format using enzyme is called an **e**nzyme-**l**inked **i**mmuno**s**orbent **a**ssay (ELISA) (Abbas, Lichtman et al. 2010). Today medical laboratories around the world are using ELISAs for routine diagnostics. Usually fully automated ELISA instruments and commercial kits are employed.

1.10 Serological tests in virus diagnostics

Serology tests are important to identify both acute and chronic viral infections. Antiviral IgM is found in the serum between 5 and 10 days post-infection and is undetectable two to four months later. Thus specific IgM antibodies are not found in persistent or chronic infections. IgM and IgA cannot cross the placental barrier. Detecting them in newborns indicates congenital infection (Mahy and Ter Meulen 2007; Yinon, Farine et al. 2010).

Recent infections can also be detected by the analysis of paired serum samples, the first sample obtained in the acute phase of the disease and the second two weeks later. A four-fold or greater rise in specific IgG antibody titer strongly indicates an acute infection (Cremer, Cossen et al. 1982; Mahy and Ter Meulen 2007).

The set of antibodies against different polypeptides of a given virus indicates the stage of infection. For instance, antibodies against three HBV antigens can be found: the anti-HBs to the HBsAg, anti-HBc to **h**epatits **B** **c**ore **a**nti**g**en (HBcAg), and anti-HBe to **h**epatitis **B** **e** **a**nti**g**en (HBeAg). In chronic HBV infections, the presence of anti-HBc and the absence of anti-HBs is found, while the presence of anti-HBc, anti-HBs and anti-HBe indicate recovery from infection (Chang and Lewin 2007).

1.11 Limitations of the current serological tests

Mostly, the antibody is directed against an epitope within the virus' antigen. **C**onventional **s**erological **t**echniques (CST) detect the presence or absence of serum antibodies against an antigen. In doing so they do not provide information on antibodies against individual peptide epitopes. Identifying antibodies at the epitope level is of great significance because not all antibodies are neutralizing and able to stop the infection. Besides, each epitope has its own function. For instance, there are epitopes responsible for binding the virus to the cell surface, others assist the virus into the host cell (Burton 2002). Binding antibodies to other epitopes may not have a functional consequence. Information on such individual epitopes is missed when performing diagnostics CST. Furthermore, the identification of distinct epitopes expressed during the virus' lifecycle may make it possible to detect epitopes specific for a distinct phase of the disease, track disease progression, and lead to a better understanding of the viral life cycle. This information may be exploited for new treatment approaches (Vigil, Davies et al. 2010).

Furthermore, highly immunogenic parts of viruses are often less genetically conserved. Virus types are characterized by multiple viruses of the same gene, e.g. **h**uman **p**apiloma **v**irus (HPV) encodes only eight proteins but so far more than 200 types have been described (Mahy and Ter Meulen 2007; Vigil, Davies et al. 2010). These individual HPV types prefer distinct target cells and have different pathogenic potentials. The high mutation rate of the hemagglutination gene of influenza viruses enables the virus to escape the immune response to an earlier variant and may lead to the emergence of highly pathogenic influenza virus strains (Mueller, Renzullo et al. 2010). Testing for different virus variants with CST is very difficult since a separate test must be run for each variant. Such tests consume much serum and can hardly be run for individual patients. Moreover the unique pattern of activity of each patient is missed. These disadvantages of CST can be only overcome by drastic miniaturization and parallelization of serological assays.

High throughput screening of thousands of peptides in parallel make possible the discovery of new diagnostic serological markers as well as permit the detection of antibodies to multiple antigens of different pathogens in the same sample. A serum antibody titer may not contain enough information about the potential protection of a vaccinated individual. Profiling the antibody response to unique neutralizing epitopes would allow for a better protection. Finally, The detection of antibodies against functionally important virus epitopes in patients recovering from a viral disease may identify new antigens for future vaccines.

1.12 Aim of the study

As explained above, the CST cannot detect important and diagnostically relevant features of the individual antibody response. This can only be achieved by peptide array technology. Such a technique would consume minute volumes of serum and could be performed in parallel with a large number of samples. Moreover, further development of this technique would increase diagnostic speed, reduce size and price of the required equipment as well as consume much less reagent and produce less waste. To explore the potential of this new area of research and development this thesis evaluates peptide microarrays deduced from HBV and HIV sequences as well as random peptide library using monoclonal antibodies and sera from vaccinees and patients.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Material were supplied by the following distributors/companies:

BD, Heidelberg:

Difco™ skim milk

Enzo Life Sciences, Loerrach (Biomol):

5-Bromo-4-chloro3indolylphosphate toluidine salt (BCIP)

Jackson ImmunoResearch, USA:

Normal goat serum, normal rabbit serum

Merck, Darmstadt:

Hydrochloric acid 37%, magnesium chloride, monopotassium phosphate, monosodium phosphate, N-N dimethylformamid, Thiazolyl Blue Tetrazolin Bromide (MTT)

Roth GmbH, Kalsruhe:

Albumin fraction V, Base Tris, Citric acid, potassium chloride, sacchsrose, sodium chloride, sodium hydroxide

Sigma-Aldrich, Munich:

10 x blocking buffer, ethanol (absolute, Spectranal), Tween20

Whatman, USA:

Whatman protein microarray blocking buffer

2.1.2 Buffers and solutions

The composition of the buffers and solutions were:

Bovine **s**erum **a**lbumin (BSA)	3g Albumin fraction V in 100ml PBS
Citrate **b**uffered **s**aline (CBS)	8.0 g of NaCl , 0.2 g of KCl , 10.51 g of citric acid (monohydrate) , pH to 7.0 with NaOH
Color **d**eveloping **s**olution (CDS)	60 mg BCIP, 50 mg MTT, 10 ml CBS DMF (1 ml absolute and 1 ml 70% [v/v] in H_2O), 50 µl $MgCl_2$ (1 M)
Membrane **b**locking **s**olution (MBS)	160ml T-TBS pH 8.0, 40ml 10xblocking buffer, 10g Saccharose
PBS-Buffer (1x)	10 mM Na2HPO4, 1.8 mM KH2PO4 pH 7.3, 140 mM NaCl, 2.7 mM KCl
Tris **b**uffer **s**olution (TBS)	6.2g base Tris, 0.2 KCL, 8.0 g Nacl, ca.3.8ml HCL, pH 7.0
Tween-**T**ris **b**uffer **s**olution (T-TBS)	6.2g base Tris, 0.2 KCL, 8.0 g Nacl, ca.3.8ml HCL, 500µl Tween 20

2.1.3 Antibodies

2.1.3.1 Primary antibodies

2.1.3.1.1 Polyclonal antibodies

Dr. Angela Uy (Universitätsmedizin Göttingen) provided 72 sera samples from HBV-infected patients (table **S**1), 22 serum samples from individuals vaccinated with Engerix-B-vaccine (table **S**2), and 20 sera from HBV-unvaccinated and HBV negative individuals. An additional 26 serum samples from individuals vaccinated with Engerix-B vaccine (table **S**3) were obtained from Prof. Dr. Wolfram Gerlich (Institute of Medical Virology, University of Giessen). Prof. Dr. Michael Roggendorf

(Insitute of Virology, University Clinic, Essen) supplied serum samples from six HBV-infected patients vaccinated with Bio-Hep-B-vaccine (table **S**4).

2.1.3.1.2 Monoclonal antibodies

The chips were reacted with the monoclonal antibodies listed in table 1.

Table 1: List of monoclonal antibodies.

Designation	Target antigen	Source
2c	Herpes simplex virus (HSV)	Prof. Dr. Michael Roggendorf, Institute of Virology, University Clinic, Essen
ARP301(221) ARP3051 (SR3 (4D7/4)) EVA3012 (4G10) EVA3046 (IIIB-V3-01) EVA3047 (IIIB-V3-13/IIIB-V3-34) EVA3048 (IIIB-V3-21) EVA3063 (2F5) EVA328 (60.1.1) EVA329 (136.1) EVA332 (187.2.1)	HIVenv	The **E**uropean **V**accine Against **A**IDS (EVA) program, Center of AIDS Reagent, attached to the **N**ational **I**nstitute for **B**iological **S**tandards and **C**ontrol (NIBSC), UK
HB1 HB3 HB7 MA18/7 Q19/10 C20/02 2-11B1	HBsAg	Prof. Dr. Wolfram Gerlich, Institute of Medical Virology, University Medical Center, Giessen

2.1.3.2 Polyclonal secondary antibodies

Secondary antibodies are listed in table 2.

Table 2: List of secondary antibodies.

Designation	Source
$Cy^{TM}3$-conjugated Streptavidin, $Cy^{TM}3$-conjugated IgG fraction monoclonal mouse anti-biotin, $Cy^{TM}5$-conjugated AffiniPure goat anti-mouse IgG (H+L), $Cy^{TM}5$-conjugated AffiniPure goat anti-human IgA + IgM + IgG (H+L), $Cy^{TM}5$-conjugated AffiniPure $F(ab')_2$ fragment goat anti-human IgG (H+L)	Jackson ImmunoResearch laboratory, USA (Dianova, Hamburg)
Goat anti-mouse IgG labeled with alkaline phosphatase	Sigma-Aldrich, Munich

2.1.4 Microarray and miniarray chips

Dr. Ronald Frank and Ulrike Beutling (Department of Chemical Biology, Helmholtz Centre for Infection Research (HZI), Braunschweig, Germany) donated the microarray chips. Peptides were synthesized via SPOT synthesis (Frank 1992), passed through the SC^2 process (Dikmans, Beutling et al. 2006) and spotted onto glass microscope slides. The miniarray chip contained between 500-1000 spots measuring ca. 750 µm in diameter. The microarray chip comprised 5000-10 000 spots with spot size between 200-250 µm (Dikmans, Beutling et al. 2006).

2.1.4.1 HBV miniarray chip

One-hundred thirty 15-mere peptides overlapping by twelve amino acids representing the entire aa sequence of the HBsAg genotype A2 (PubMed accession number X02763) were synthesized and spotted onto glass microscope slides to produce the HBV miniarray. Its layout is shown in figure 4. An Excel file containing the coordinates of the HBV miniarray chip is given on the supplementary CD with the file name ´´HBV miniarray´´.

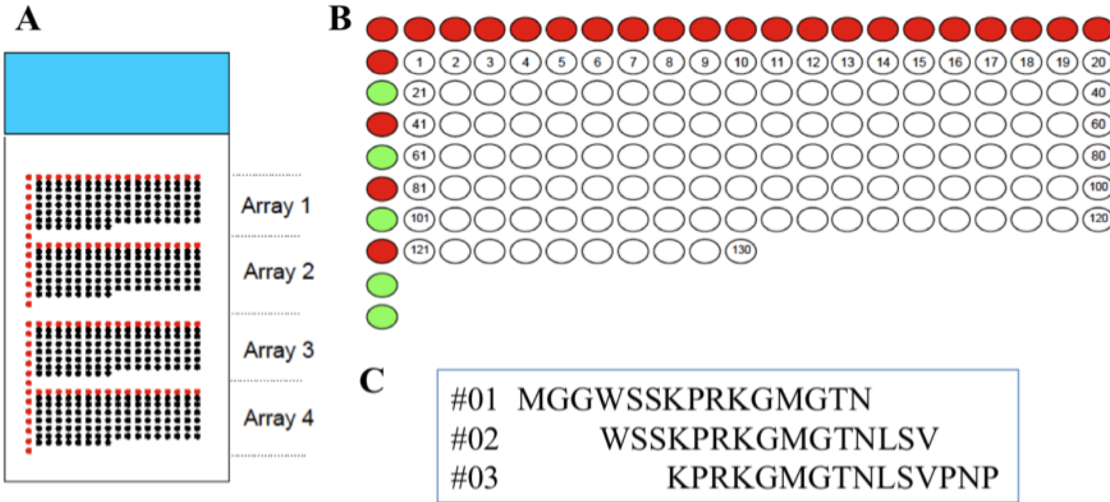
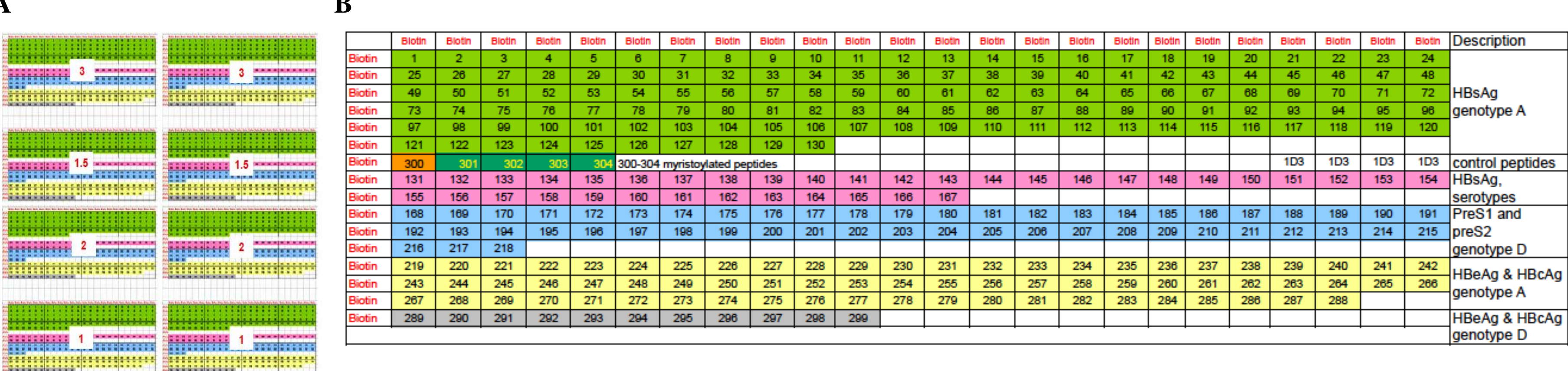


Figure 4: Layout of the HBV miniarray chip. (A) The whole chip contains four identical arrays. (B) Arrangement of spots on each array (red: biotin positive-control, green: beta-alanine negative-control, and uncolored circles: HBsAg aa sequence). (C) the aa sequences of the first three peptides to show the overlap of 12 aa (Ulrike Beutling, personal communication).

2.1.4.2 HBV microarray chip

The HBV microarray chip contains 12 identical arrays of 348 spots each (figure 5A). Two-hundred-ninety-nine of them are 15mere peptides overlapping twelve aa, which representing the entire aa sequence of HBsAg genotype A2 (PubMed accession number X02763), preS1 and preS2 of genotype D (PubMed accession number X02496), HBsAg serotypes and HBeAg and HBcAg. In addition, four peptides as synthesis control, 40 biotin spots as a positive control, and HBsAg myristoylated N-terminus peptides (figure 5B) were added to the chip. The Excel file named ´´HBV microarray´´ containing the coordinates of the HBV microarray chip is provided on the supplementary CD.



	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Biotin	Description
Biotin	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	HBsAg genotype A
Biotin	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	
Biotin	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	
Biotin	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	
Biotin	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	
Biotin	121	122	123	124	125	126	127	128	129	130															
Biotin	300	301	302	303	304	300-304 myristoylated peptides															1D3	1D3	1D3	1D3	control peptides
Biotin	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	HBsAg, serotypes
Biotin	155	156	157	158	159	160	161	162	163	164	165	166	167												
Biotin	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	PreS1 and preS2 genotype D
Biotin	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	
Biotin	216	217	218																						
Biotin	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	HBeAg & HBcAg genotype A
Biotin	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	
Biotin	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288			
Biotin	289	290	291	292	293	294	295	296	297	298	299														HBeAg & HBcAg genotype D

Figure 5: Layout of the HBV microarray. (A) The HBV microarray chip contains twelve arrays arranged in the same manner, however, five different peptide concentrations were applied. The variation in the peptide concentration of the spotting solution is given in red in the insert. (B) Layout of a single array of the HBV chip. HBsAg genotype A are given in green, HBsAg myristoylated N-terminal peptides in dark green, serotypes of HBsAg peptides in pink, peptides of the preS1 and preS2 region of HBsAg genotype D in blue, genotype A peptides of HBeAg and HBcAg in yellow, HBeAg and HBcAg genotype D peptides in grey, and biotin positive-control spots in red (Ulrike Beutling, personal communication).

2.1.4.3 HIVenv microarray chip

The HIVenv microarray chip comprise 4896 spots arranged in 16 identical arrays (figure 6A), each containing the same 282 overlapping 15-mere peptides representing the 856 aa of the gp160 of HIV-1 (B.FR.83.HXB2_LAI_IIIB_BRU.K03455, www.hiv.lanl.gov). Thirty spots with cellulose-conjugated biotin serve as a positive control and 6 with cellulose conjugated beta-alanine as a negative control (figure 6B). An Excel file containing the coordinates of the HIVenv microarray chip is given on the supplementary CD with the file name ´´HIVenv microarray´´.

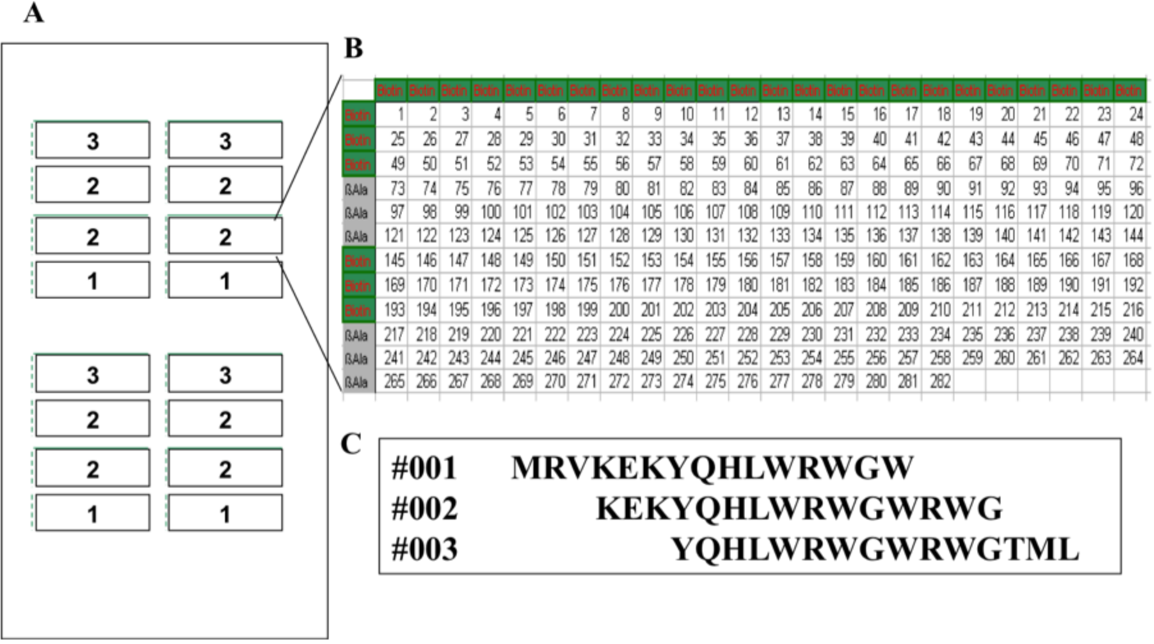


Figure 6: Layout of the HIVenv microarray chip. (A) The chip contains 16 arrays. Three different peptide concentrations were applied. The variation in the peptide concentration of the spotting solution is given. (B) Arrangement of spots on each array, green: biotin positive-control; gray: beta-alanine negative-control; and numbered squares: HIV gp160 aa sequence. (C) The aa sequences of the first three peptides to show the overlap of 12 aa (Ulrike Beutling, personal communication).

2.1.4.4 Random peptide library

The random peptide library contains 4608 15-mere peptides (4608_RPL). The 4608 peptides were arranged in 48 quadrants (figure 7A). Each quadrant contained 96 peptides arranged in a 12 x 8 pattern similar to a micro-titer plate (figure 7B). A replicate of each spot was placed below the original one (figure 7B and 7C). An Excel program called "Seek-Peptide" was developed for easy sequence identification of reactive peptides

(figure 8). To identify the peptide sequence of a spot, the coordinates are entered into the Seek-Peptide program. The quadrants are abbreviated to Q1-Q48. The Y position of each spot is then given a capital letter while the x position is identified by a number e.g. Q1E7 (figure 7D). The Excel data sheet for the 4608_RPL was placed on the supplementary CD under the title "4608_RPL".

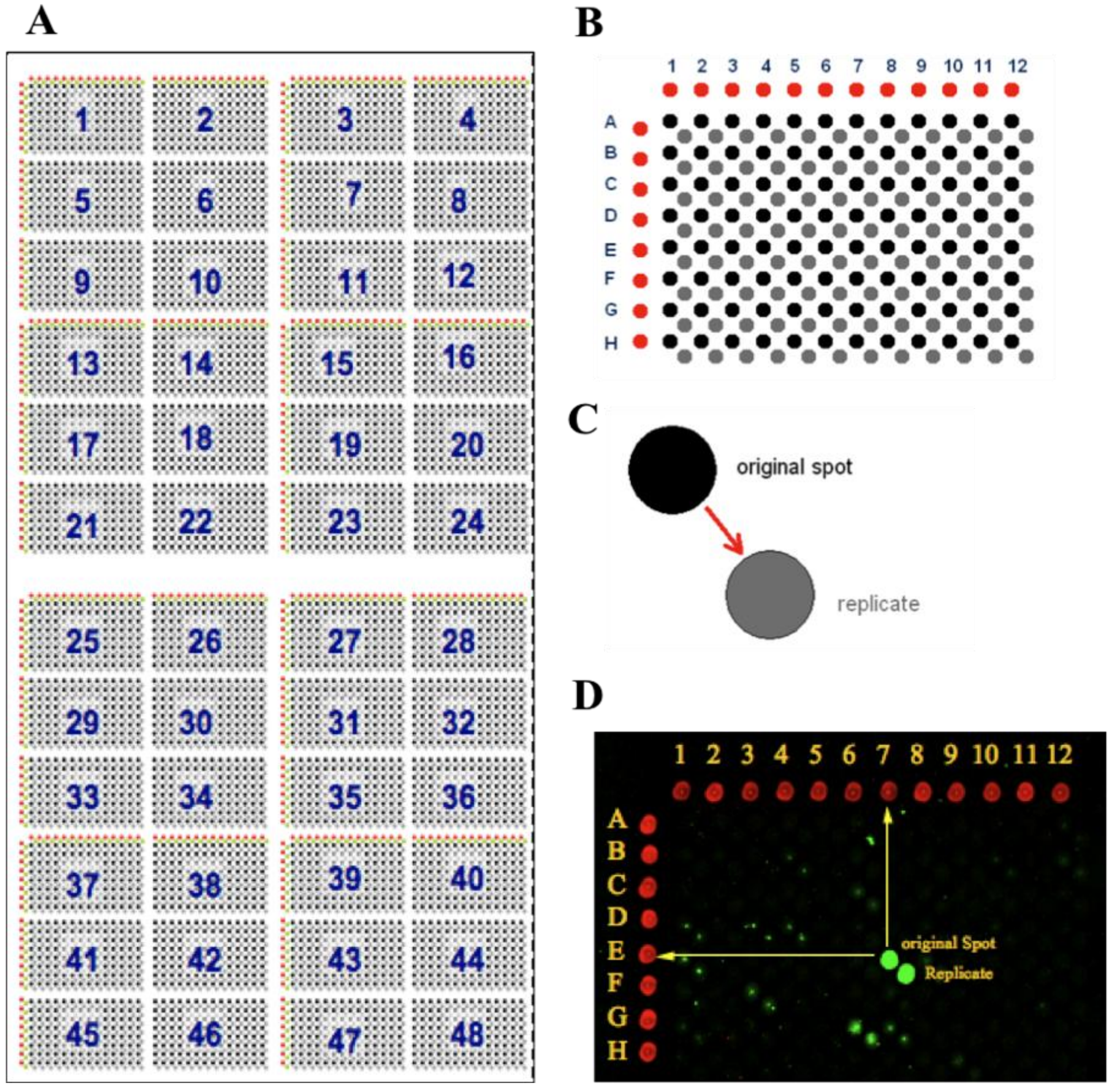


Figure 7: Layout of the 4608_RPL. (A) The 4608_RPL microarray chip contains 48 quadrants. (B) Layout of one quadrant; red, biotin positive-control; black, 15-mere random peptides; grey, peptide replicate. (C) Position of spot and its replicate. (D) Localization of positive-reacting peptide within a quadrant (Ulrike Beutling, personal communication).

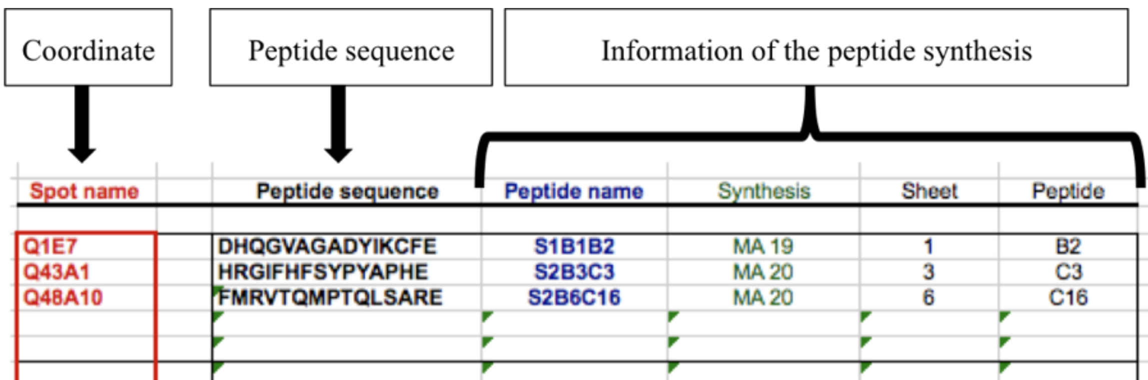


Figure 8: Structure of the Seek-Peptide program. To identify the sequence of a spot, a coordinate is entered into the red left hand column and the enter button is pressed on the PC keyboard. The program then provides the peptide sequence and synthesis information.

2.1.5 Equipment

The following equipment was used:

Agilent microarray scanner	Agilent Technologies, Inc., USA
Balance TE612	Sartorius, Göttingen
DFL shaker	Schuett24GmbH, Göttingen
HeraFreeze	Thermo scientific, Langenselbold
Laminar flow Hera Safe	Thermo scientific, Langenselbold
Magnetic Stirrer MR3000	Heidolph GmbH, Schwabach
Multifluge3$_{L-K}$	Thermo scientific, Langenselbold
pH-Meter	Schütt Labortechnik, Göttingen
Pipetus ®	Hirschmann Laborgeräte GmbH, Eberstadt
Rotilabo® Desiccator	CARL ROTH GmBH, Karlsruhe
Thermomixer Compact	Eppendorf Ag, Hamburg
Varioklav, Autoclave	Thermo scientific, Langenselbold
Vortex Genie 2™	Bender & Hobein AG, Zurich, Switzerland
Water bath SW22	Julabo GmbH, Seelbach

2.1.6 Software

Software obtained from:

Agilent Feature Extraction	Version 7.5, Agilent Technologies, Inc., USA
Endnote	Version X3, Thomson ISI Research Soft, Carlsbad, CA
MEME	Motif-based sequence analysis tools, http://meme.nbcr.net/meme4_5_0
Microsoft® Office Excel	Version 2008 for Mac, Microsoft Corporation
Microsoft® Office Word	Version 2008 for Mac, Microsoft Corporation
Muscle	Service of **E**uropean **B**ioinformatics **I**nstitute (EBI), www.ebi.ac.uk/Tools/msa/muscle/
Swiss-Pdb Viewer	The Swiss Institute of Bioinformatics, Version 4.0, http://spdbv.vital-it.ch/
Seek-Peptide	Ulrike Beutling (Department of Chemical Biology, HZI, Braunschweig

2.1.7 Database

The NCBI-Databank provided protein, nucleotide, and genomic sequences (http://www.ncbi.nlm.nih.gov/). To search for epitopes, the Immune Epitope Database (http://www.immuneepitope.org/) was used. HIV sequences were identified *via* the HIV database (http: //www.hiv.lanl.gov/).

2.2 Methods

2.2.1 Microarray chip screening

2.2.1.1 General screening procedures

The microarray chip was washed with absolute ethanol for 3 min and then with TBS three times for 3 min. After incubation with blocking buffer for one hour, the chip was washed with T-TBS for 3 min. Thereafter, the primary antibody diluted 1/100 in blocking

buffer was added onto the chip. The chip was incubated in a humidified chamber at room temperature for 3.5 hours. The chip was then washed 3 times for 5 min with T-TBS. To visualize the binding of the primary antibody, solution containing Cy3-conjugated streptavidin and Cy5-conjugated goat anti-species IgG (Jackson Immunoresearch Laboratories) was added onto the chip. The chip was kept in a humidified chamber at room temperature for 1.5 hours. Subsequently; the chip was washed twice for 5 min with T-TBS, 3 times with distilled water for 5 min, and dried before screening with an Agilent DNA microarray scanner.

2.2.1.2 Precautions during chip screening

To obtain optimum scanning results, the following instructions are critical:

- Blocking buffer must be freshly prepared.
- Volume of antibody solution added on the slide must not exceed 60 µl.
- To obtain equal antibody distribution and avoid air bubble formation, the primary antibodies as well as the secondary antibodies must be added at one end of the glass slide as one droplet. A cover slip held by a pair of forceps must be placed on the droplet at a 45° angle to the glass slide (figure 9A). The angle is then reduced gradually until the glass slide is completely covered (figure 9B).
- The cover slip must be cleaned. A washing step to clean the cover slip with 70% alcohol is necessary.

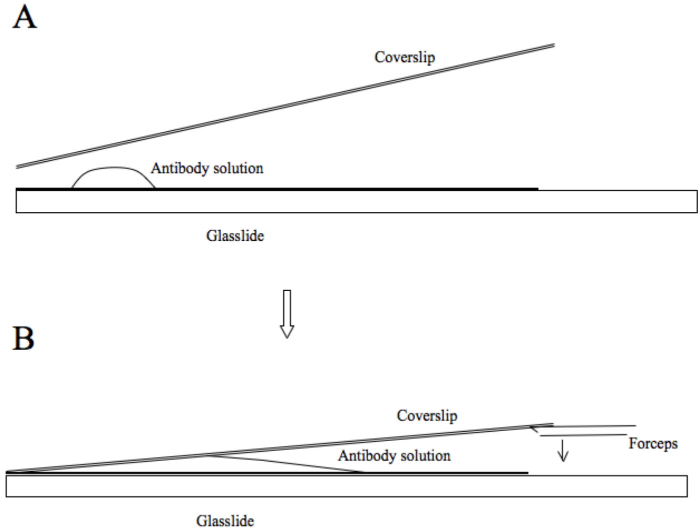


Figure 9: Applying of a cover slip to a microarray slide (Ulrike Beutling, personal communication).

- If the cover slip cannot be easily removed after incubation, the glass slide should be immersed in T-TBS for 2 min. Thus scratching off peptides from the slide surface is avoided.
- The slides must be incubated in a humidified chamber (figure 10) to avoid dust on the bottom surface of the slide.
- To avoid carrying over antibodies, use one pair of forceps per slide.

Figure 10: Incubation chamber for the microarray chip (Rotilabo®-Färbekammern, CARL ROTH GmBH, Karlsruhe).

2.2.1.3 Scanning the chip with an Agilent microarray scanner

The Agilent microarray scanner is turned on and allowed to warm up. The chip is deposited on the chip holder (figure 11). The active surface of the chip faces the cover. The chip is inserted into the carousel of the scanner. Before scanning, the Agilent Scan Control program in the attached PC must be started and adjusted to the scanning parameters (figure 12). The number of chips to be scanned is entered on the end slot field (upper right corner on figure 12). Thereafter, the scanning program is initiated by clicking on the Scan Slot field by the Scan Control program (lower right in figure 12).

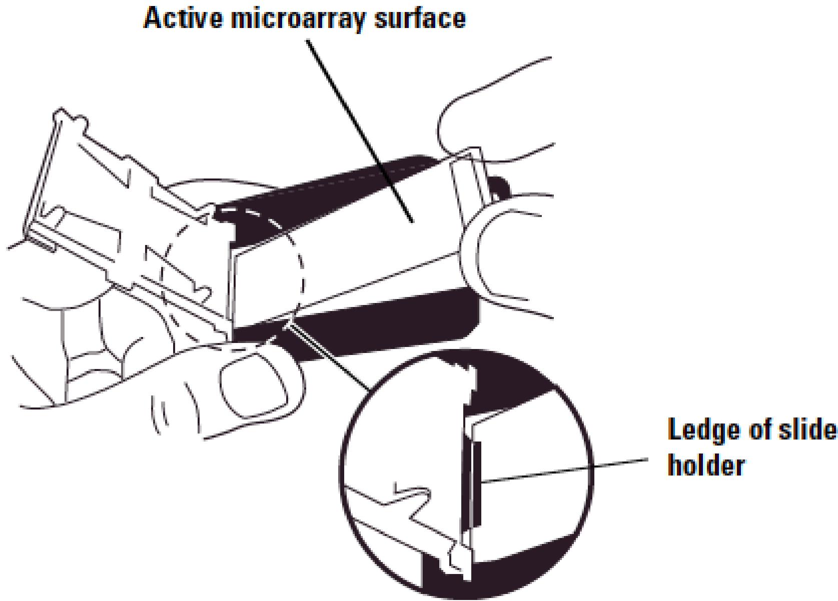


Figure 11: Placement of a microarray chip in the microarray slide holder (Agilent Technologies, Inc., USA).

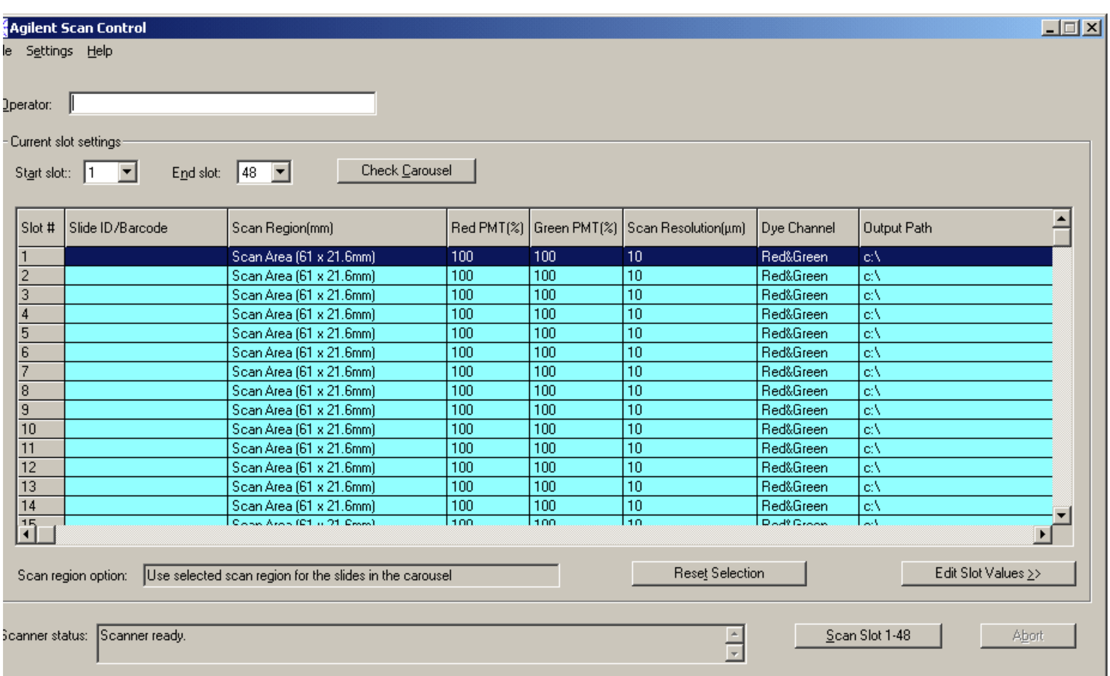


Figure 12: Agilent Scan Control program main window (Agilent Technologies, Inc., USA).

2.2.2 Filter membrane screening procedure

The filter membrane was washed with TBS 3 times each for 10 min, and then incubated with MBS at room temperature overnight. After washing with T-TBS for 10 min, the membrane was incubated with the primary antibody (40 µl primary antibody + 8 ml MBS) for 3.5 hours. The membrane was washed 3 times with T-TBS each for 10 min. Then the membrane was incubated with the secondary antibody (4 µl goat anti-mouse IgG labeled with alkaline phosphatase + 8 ml MBS) for 1.5 hours. The membrane was washed twice with T-TBS for 10 min, and then twice with CBS for 10 min. Thereafter, the membrane was incubated with CDS until the signals were visible. The membrane was washed with PBS for 30 sec, then for 10 min. Incubation and washing took place at room temperature and on a horizontal shaker.

2.2.3 Sera from monkeys immunized with HIV/SIV

The following three immunization experiments were conducted in the Infection Models Unit, German Primate Center (DPZ) under supervision of Dr. Christiane Stahl-Hennig. Serum samples were kindly provided.

2.2.3.1 Sera from monkeys immunized with DNA and modified vaccinia Ankara (MVA) as vector

2.2.3.1.1 SIV DNA and recombinant MVA vaccine constructs

Two types of immunogen were used in this experiment. First the **s**imian **i**mmunodeficiency **v**irus (SIV) DNA which was composed of two RNA- and codon-optimized DNA encoding the SIVmac239 (SIVMM239; GenBank accession no. M33262) $Pr55^{Gag}$, and the $Pr160^{Gag\text{-}Pol}$ polyprotein (SIV gag/pol), as well as the **s**imian-**h**uman **i**mmunodeficiency **v**irus (SHIV) 89.6p (GenBank accession no. U89134) envelope derivatives gp160 and gp120. Synthetic genes were created by a stepwise PCR amplification of overlapping 60-mer oligonucleotides and subcloned into the unique restriction sites *Kpn*I and *Sac*I of the pCRScript ™ Amp SK(+) cloning vector (Stratagene, La Jolla, CA). In order to obtain high level, constitutive expression in mammalian cells, the Gag-Pol as well as the gp120/160 coding regions were placed into the *KpnI* and *XhoI* restriction sites of the pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA) under the transcriptional control of the immediate-early promoter enhancer

of cytomegalovirus (CMV), which yielded the final plasmids (1) SIV gag/pol, (2) HIV-1 89.6 env120, and (3) HIV-1 89.6 env160, respectively (Stolte-Leeb, Bieler et al. 2008).

The second immunogen was the modified vaccinia Ankara (MVAgpenv) recombinants expressing the Gag-Pol protein of SIVmac239, and the HIV-1 Env protein of SHIV89.6P (KB9), under control of the modified H5 VV promoter (Stolte-Leeb, Bieler et al. 2008).

2.2.3.1.2 The regime of immunizing monkeys with DNA and MVA vector

Fourteen purpose-bred monkeys (*Macaca mulatta*) were allocated to 4 groups. The first group of monkeys (S) was immunized with SIV DNA **i**ntra**m**uscularly (IM) and **i**ntra**d**ermally (ID) at weeks 0, 8, and 16, and boosted IM and ID with an MVAgpenv at week 24 and 32. The second group of monkeys (S/M) received the same immunization as group 1 until week 24, after which they were immunized orally with MVAgpenv at week 32. The third group of monkeys received empty DNA and MVA constructs as vector controls (VC). Two animals as naïve controls (NC) remained untreated. All animals were challenged intra-rectally with the SHIV89.6P swarm virus at week 40 (table 3). From each S and S/M monkey one pre-immunization serum, 3 post-immunization sera, and one after-challenge serum were screened with the HIVenv chip. Of the VC animals, one sample was obtained at the time of challenge and two samples were tested thereafter. Two sera were tested from NC animals.

Table 3: Immunization schedule of the SIV DNA and recombinant MVA vaccination experiment (Stolte-Leeb, Bieler et al. 2008).

No. of animals	*Group*	*Weeks*		
		0, 8, 16	*24*	*32*
4	A vaccinees	SIV DNA SIV syngag-pol (0.5 mg) 89.6P synenv gp120 (0.25 mg) 89.6P synenv gp160 (0.25 mg) 0.5 mg (IM)/0.5 mg (ID)	MVAgpenv 1×10^8 (IM) 1×10^8 (ID)	MVAgpenv 1×10^8 (IM) 1×10^8 (ID)
4	B vaccinees	SIV DNA SIV syngag-pol (0.5 mg) 89.6P synenv gp120 (0.25 mg) 89.6P synenv gp160 (0.25 mg) 0.5 mg (IM)/5×0.1 mg (ID)	MVAgpenv $2 \times 5 \times 10^7$ (IM) $5 \times 2 \times 10^7$ (ID)	MVAgpenv 2×10^8 (oral)
2	C vector controls	Empty DNA 0.5 mg (IM)/0.5 mg (ID)	MVA 1×10^8 (IM) 1×10^8 (ID)	MVA 1×10^8 (IM) 1×10^8 (ID)
2		Empty DNA 0.5 mg (IM)/0.5 mg (ID)	MVA 1×10^8 (IM) 1×10^8 (ID)	MVA 2×10^8 (oral)
2	D naïve controls	Not immunized	Not immunized	Not immunized

Abbreviations: MVAgpenv, modified vaccinia Ankara expressing SIV gag/pol and HIV-1 89.6env; IM, intramuscularly; ID, intradermally.

2.2.3.2 Sera from monkeys immunized with DNA and adenovirus serotype 5 (Ad5) as vector

2.2.3.2.1 DNA and recombinant Ad5 vaccine constructs

The DNA vaccine comprised pGX10-Gag- Env, pGX10-sPol, pGX10-sVif-Nef, and pGX10-sTat-Vpx (figure 13). To construct the recombinant Ad5 expressing SIV, HIV-1 and adjuvant genes (figure 13), each gene was cloned into the pShuttle- CMV (Qbiogene, CA, USA) and then inserted by homologous recombination into pAdEasy-1 [E1/E3- deleted human Ad5 viral DNA (Qbiogene)] (Stahl-Hennig, Suh et al. 2007).

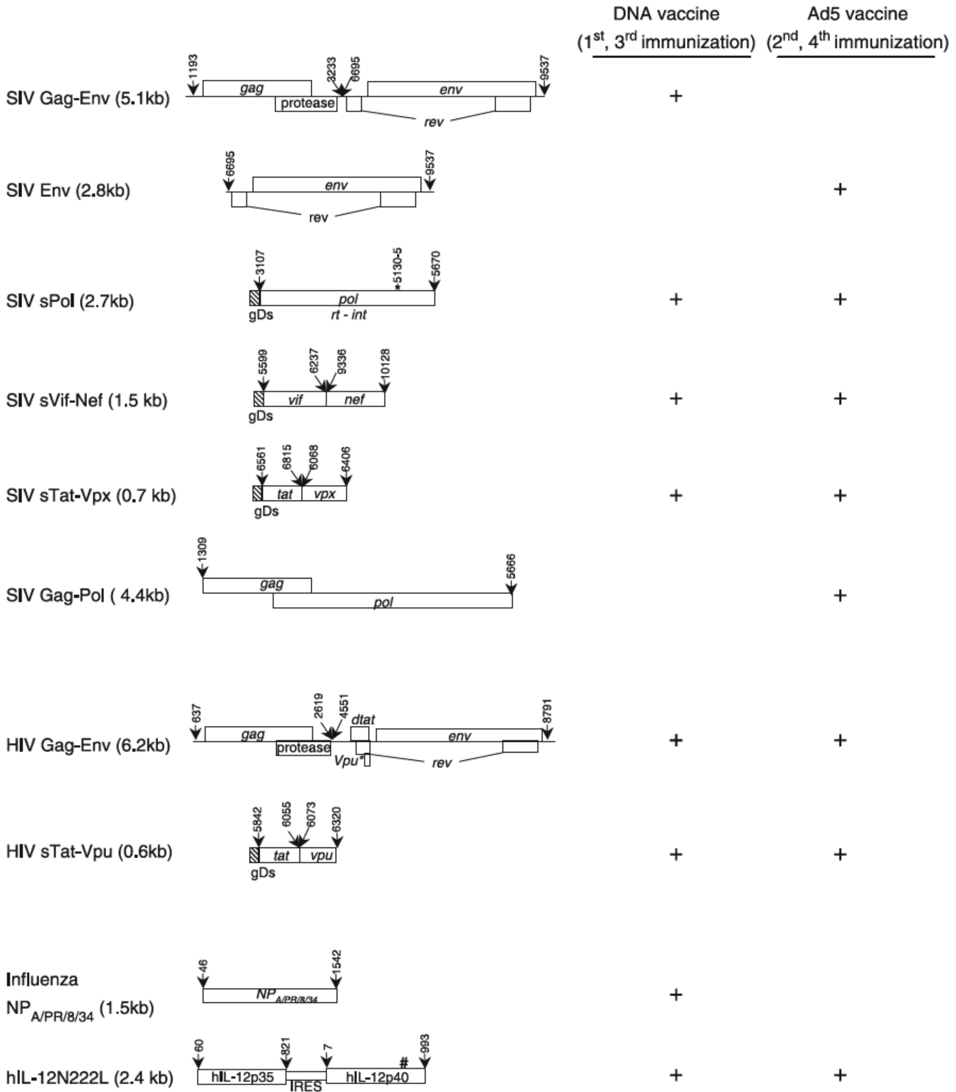


Figure 13: Schematic diagram of SIV, HIV-1 and adjuvant genes of the DNA and Ad5 vaccines. Gene sizes are indicated in parentheses (Stahl-Hennig, Suh et al. 2007).

2.2.3.2.2 The regime of immunizing monkeys with DNA and recombinant Ad5 vaccine

Two groups of six monkeys each were immunized. Group one monkeys received a DNA vaccine at week 0 and 16 and recombinant Ad5 vaccine at week 8 and 24. As controls Group two monkeys received DNA buffer and adenovirus dilution buffer. All monkeys were challenged with SHIV89.6P virus at week 40 and re-challenged with SIVmac239 at week 64. From each of the vaccinees, one pre-immunization, 5 post-immunization, and 3 post-challenge sera were screened with the HIVenv chips. Of the

control monkeys, one serum at the time of challenge and three post-challenge sera were examined.

2.2.3.3 Immunization of monkeys with single-cycle immunodeficiency virus vaccine (SCIV) and adenovirus recombinant vaccine

Four rhesus macaques were immunized **i**ntra**v**enously (IV) at week 0 with SCIV. The SCIVs were produced by transient transfection of an SIV genome that was made replication deficient by mutations in the primer binding site and a deletion of *vif*. To allow a single round of replication, the primer binding site mutations were complemented in *trans* by a matched tRNA expression plasmid in *vif*-independent 293 producer cells. After administration to the vaccinees, the SCIVs can undergo only a single round of replication, leading to the production of noninfectious virus-like particles *in vivo*. At week 8, the four macaques were immunized IM with recombinant adenovirus expressing the SIV envelope. Then, the macaques were challenged at week 20 with SIVmac239 by the tonsillar route (Stahl-Hennig, Kuate et al. 2007). Only sera obtained directly before challenge were screened with HIVenv chips.

3 Results and discussion

3.1 Method Development

3.1.1 Blocking buffer

Pretreatment of the microarray chip with a blocking buffer (BB) is an important step, since it will minimize non-specific binding and increase the signal-to-noise ratio (S/N) (Collett, Cho et al. 2005). Three blocking buffers were compared on three HBV miniarrays: 4% **s**kimmed **m**ilk (SM) in T-TBS, 3% BSA in PBS, and **W**hatman protein **m**icroarray blocking **b**uffer (WMB). The blocked miniarrays were then tested with the same human serum from an HBV-vaccinated individual. This serum reacted with spot numbers 96, 97, 98, and 99 (figure 14). The SM produced the lowest background and the highest S/N ratio. The reactivity was lost when WMB was used. SM was used as the BB for most of the work presented here. Later it was replaced with MBS containing casein, and saccharose in T-TBS. Its blocking activity was equivalent to SM.

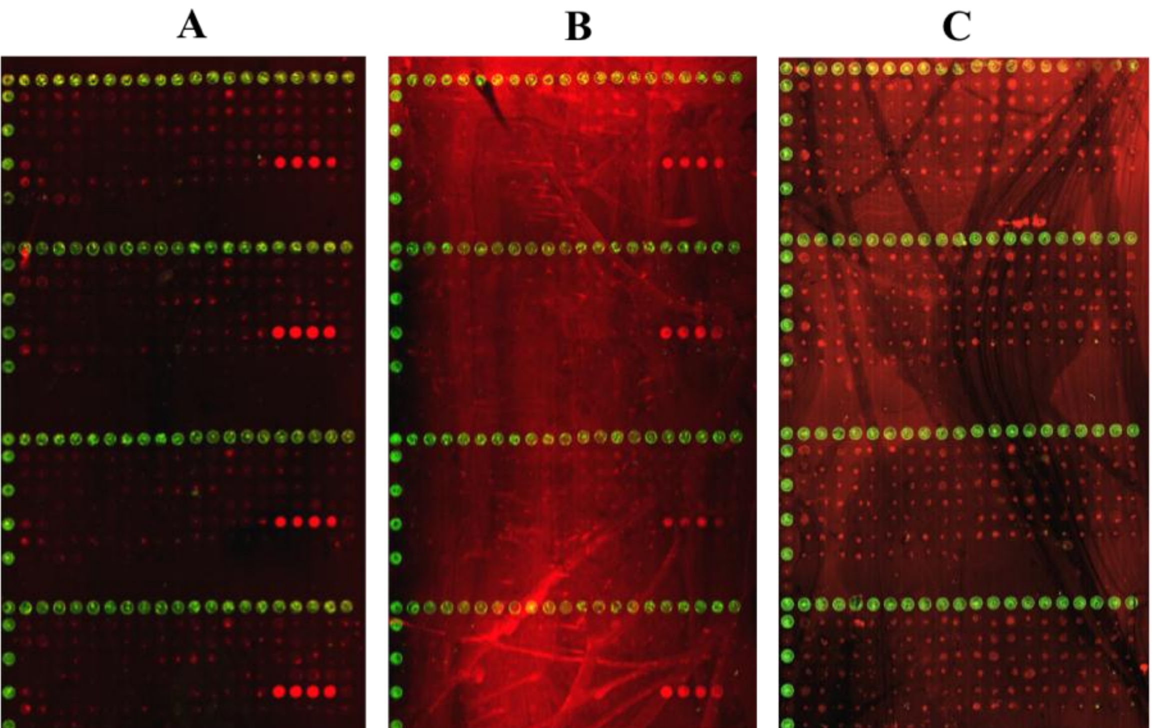


Figure 14: Comparison of blocking buffers. (A) SM, (B) BSA, and (C) WMB.

3.1.2 Peptide-cellulose conjugate (PCC) stock solution

The peptides printed onto the microarray chip were synthesized by SPOT technology allowing for direct synthesis of peptides onto a cellulose membrane (Frank 1992). The peptide-containing cellulose spots were then punched out into a deep-well microtiter plate. The cellulose disc was dissolved in a trifluoroacetic acid (TFA) cocktail. This treatment solubilized the cellulose fibers while the peptide was still attached to small cellulose fragments. The PCC was precipitated with ether to remove the TFA however incompletely. PCC precipitate was dissolved in dimethyl sulphoxide (DMSO). To produce a microarray chip, PCC solution was printed onto glass microscope slides (Dikmans, Beutling et al. 2006). PCC stock solutions were kept at -20°C for later printing of additional chips.

Over a year, HBV chips from different batches were used to screen mAbs and human sera. It was noticed that the stock solutions kept at -20°C underwent an aging process, i.e. there was a reduced sensitivity of chips printed later. To examine this process, the reactivity of peptides printed directly after synthesis was compared to those kept at -20°C and printed after one year. For this comparison, peptides reacting with two mAbs (MA18/7 and HB1) were selected. Chips were printed with freshly synthesized peptides and those kept for one year (figure 15). After reaction with the respective two mAbs, marked differences were seen between peptides printed directly after synthesis and those printed after one year. Thus printing directly after synthesis of peptides produces an optimum S/N ratio. Furthermore, only high concentrations of the stored peptides led to positive signals. A reduction in peptide reactivity might be explained by reactivation of a remnant of TFA upon thawing, which could reduce the ability of the PCC to stick to the glass surface.

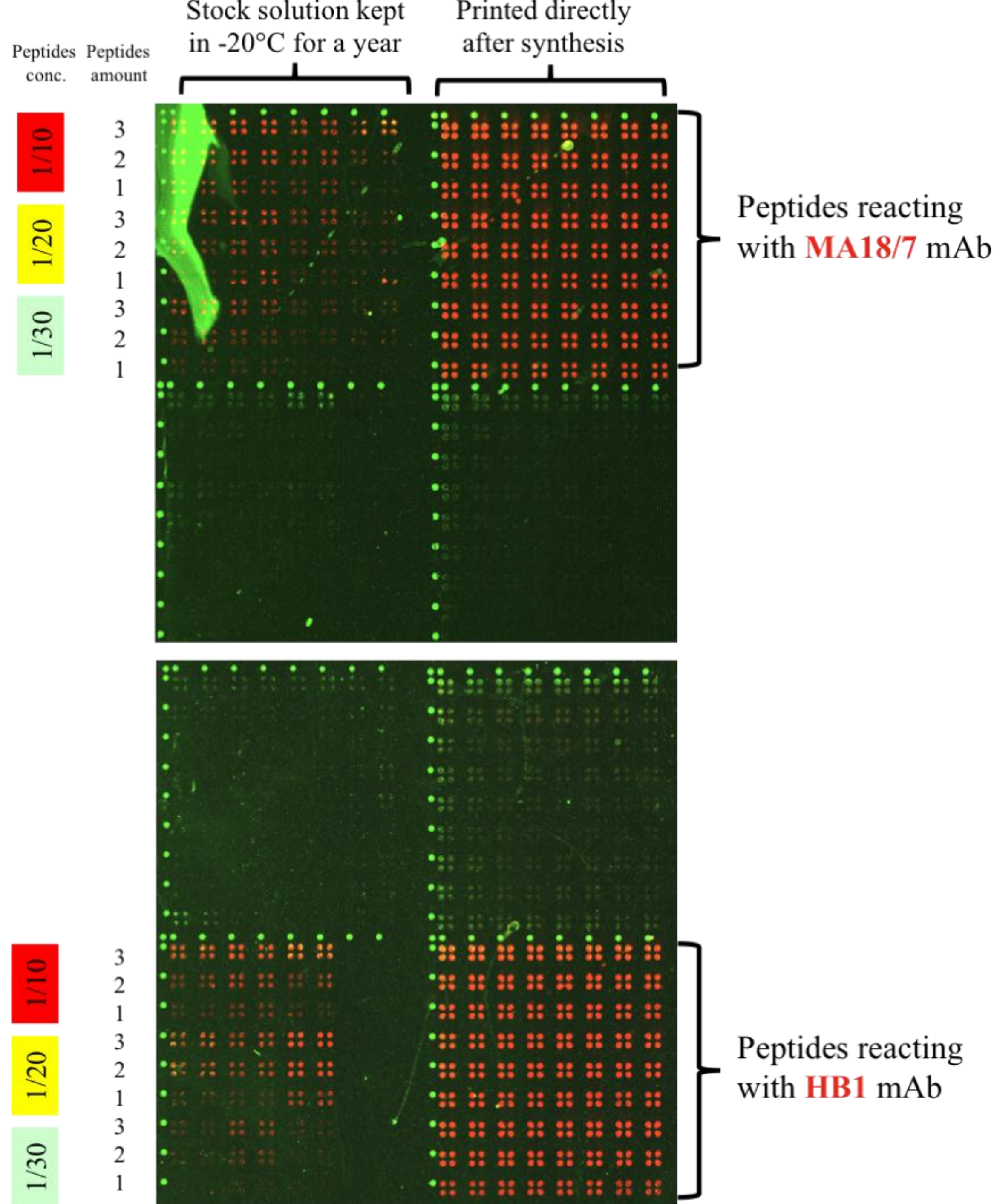


Figure 15: Comparison between the reactivity of peptides printed directly after synthesis and those after kept in -20°C for a year. As indicated on the left hand side, three different concentrations and amounts were applied.

3.1.3 Storage conditions for printed slides

These experiments were performed by Ulrike Beutling, Department of Chemical Biology, HZI, Braunschweig, Germany. Forty-seven slides were spotted with biotin and a peptide (sequence: NYGKYE) reacting with the ID3 mAb. Slides were stored at different conditions shown in table 4. After 195 days, two slides from each group were incubated with the ID3 mAb. Binding was visualized with secondary antibodies (Cy5-goat-anti-mouse antibody, and Cy3-Streptavidin) (table 5).

Table 4: Different storage conditions of the slides.

Storage conditions	Slides numbers	Storage temp.	Gas[2]	Drying bag used/number[1]	Sealed in plastic bag	Preservation of reactivity
At room temperature	21,22,35,45,55,65	20°C	---	---	1x sealed	Low
At room temperature + drying bags	42, 43	20°C	---	yes 2 pieces	---	Low
Refrigerator + drying bags	23,24,36,46,56, 66,44	4°C	---	yes 2 pieces	1x sealed	Medium
Deep freezer (-20°C) + drying bags	25,26,37,47,57,67	-20°C	---	yes 2 pieces	1x sealed	High
Refrigerator + drying bags + argon	27,28,40,50,60,61	4°C	Argon	yes 2 pieces	2x sealed	High
Drying oven [3] + drying bags	29,30,38,48,58,68	50°C	---	yes 2 pieces	---	Low
At room temperature + drying bags + argon	33,34,41,51,62,63, 52,54	20°C	Argon	yes 2 pieces	2x sealed	Medium
Desiccators [3] + drying bags + argon	31,32,39,49,59,64	20°C	Argon	yes 2 pieces	---	Medium

[1] The drying bags were separated from the printed slides by an unprinted microscope to avoid dust coming into contact with the printed slides surfaces.

[2] When argon was used, the slide boxes were filled with the argon and closed (not sealed). They were then put in a plastic bag, which was also filled with argon and sealed with a bag sealer. Again, slides contained in the box and the bag were put into a second bag filled with the argon.

[3] The boxes in the desiccators and in the drying oven were not sealed with plastic bags.

Conclusions on the storage conditions experiment are shown in table 5:

The poorest results were from slides stored in the drying oven and at room temperature (regardless of with or without drying bag). The best results were from ones were stored in the refrigerator with drying bags and under argon or in the deep freezer with drying bags. Both showed similar spot intensities after storage. Generally the peptide spots seemed to be more sensitive to storage conditions than the biotin spots (on the poorest slides, the peptide spots disappeared earlier than the biotin spots).

Table 5: Influence of different storage conditions on the signal intensities.

Storage conditions	Intensity aligned to (3000/30000)	
At room temperature	_21	_35
At room temperature + drying bags	_42	_43
At room temperature + drying bags + argon	_33	_41
Drying oven + drying bags	_29	_38
Refrigerator and drying bags	_23	_36
Refrigerator + drying bags + argon	_27	_40
Deep freezer (-20°C) + drying bags	_25	_37
Desiccator + drying bags + argon	_31	_39

3.2 Monoclonal antibodies

3.2.1 General remarks

As descried in the Material section, the HIVenv chip is composed of 16 identical arrays, the HBV chip contains 12 identical arrays, and the HBV miniarray comprises four identical arrays. The only difference between the arrays within the same chip is the amount of peptide per spot. The HIVenv chip was stained with EVA3047 mAb (figure 16). All arrays displayed the same result except for arrays 7, 8, 15, and 16. The weak signal there was to be expected since these spots contain the lowest amount of peptides. Figure 17 shows only one of the 16 arrays. All further results will be represented in the same way and aa sequences of the reacting peptides are shown and the shared aa sequences colored red.

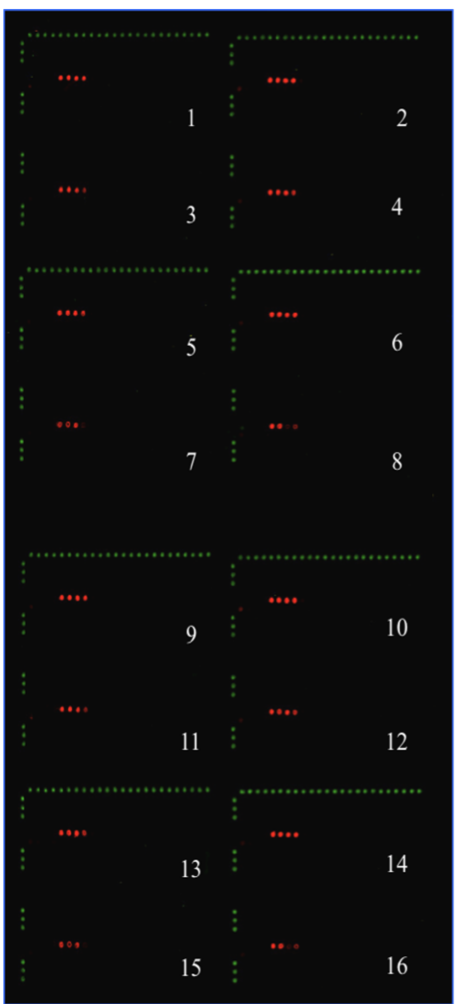


Figure 16: Testing the HIVenv chip with EVA3047. The arrays are numbered 1-16.

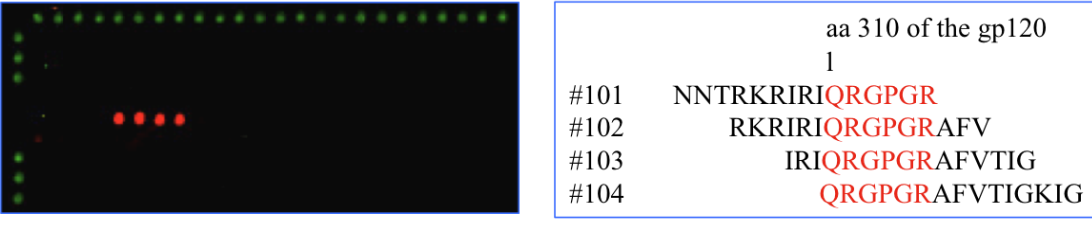


Figure 17: EVA3047-reactive peptides on the HIVenv chip. Left, array one of HIVenv chip (figure 16). Right, corresponding aa sequences of the reacting spots. The target sequence is shown in red comprising the aa 310-315 of the gp160 sequences.

3.2.2 Reactivity of monoclonal antibodies with HIVenv and HBV chips

3.2.2.1 Reactivity of the mAbs (EVA3012, EVA329, EVA3063 and EVA328) with the HIVenv chip

The four HIVenv mAbs, EVA3012, EVA329, EVA3063 and EVA328 from Dr. A von Brunn, Dr. H Katinger, Dr. C Thiriart and Dr. C Bruck were provided by the EVA Program, Center of AIDS Reagent attached to the NIBSC, UK. The available information about these mAbs and their targets is listed in table 6. These mAbs were screened using the HIVenv chip to reproduce the results obtained by others.

EVA3012 reacted with the four peptides (102-105) of the HIVenv chip (figure 18A). The target of EVA3012 defined as the shared sequence of the four peptides is the PGRAFV (aa 313-318 of gp120). Earlier, the target of this mAb was identified with competitive ELISA to comprise the 15 aa RIQRGPGRAFVTIGK (table 6) (von Brunn, Brand et al. 1993). Likewise, using the scanning chip, the target sequence of EVA329 mAb was reduced from 21 to 6 aa (table 6 & figure 18B) (Thiriart, Francotte et al. 1989).

The target sequence of mAb EVA3063 obtained with the HIVenv chip with EVA3063 was LELDKW (aa 661-666 of gp120) (figure 18C). In earlier work, using a peptide ELISA, a similar sequence was identified with shifted one aa to N-terminus (table 6) (Buchacher, Predl et al. 1994). The differences of the aa sequences described as targets is explained by the fact that our HIVen chip contained 15-mere with 12 aa overlapped with the neighboring.

Table 6: Known information on the HIVen mAbs and reactivity with the HIVenv chip.

Designa-tion	Isotype	Immunogen	Specificity*	Reference	Target sequences+
EVA3012 (4G10)	Mouse (BALB/c) / Ag8653 / IgG1	HIV-1 gp120 (aa 308-322) fused to HBcAG particles	Reacts in ELISA with V3 loop, peptide ARP734 RIQRGPGRAFVTIGK, and with purified gp160.	(von Brunn, Brand et al. 1993)	aa 313-318 of gp120 (PGRAFV)
EVA329 (136.1)	Mouse (BALB/c) / Sp2 / IgG2ak	Recombinant HIV-1 gp160 produced in yeast cells	HIV-1 gp160/gp120 by RIPA / Immunoblot / ELISA. Target sequence described aa 362-381FKQSSGGDPEIVTHSFNCGGE	(Thiriart, Francotte et al. 1989)	aa 376-381 of gp120 (FNCGGE)
EVA3063 (2F5)	HIV-1 infected human / CB-F7 IgG1k	Recombinant human Mab produced in CHO cells	HIV-1 gp41, Target sequence described aa 662-667 ELDKWA	(Buchacher, Predl et al. 1994)	aa 661-666 of gp 41 (LELDKW)
EVA328 (60.1.1)	Mouse (BALB/c) / Sp2 / IgG2ak	Recombinant HIV-1 gp160 produced in yeast	HIV-1 gp160/gp41 by RIPA, Immunoblot., Target sequence described aa 362-381 FKQSSGGDPEIVTHSFNCGGE)	(Thiriart, Francotte et al. 1989)	aa 733-735 of gp 41 (EGG)

*The target sequences of mAbs detected by other authors are colored blue. + The target sequences of mAbs obtained by the HIVenv scanning are colored red.

The result obtained with fourth mAb EVA328 was surprising. Its target sequence on our HIVenv chip was EGG (aa 733-735 of gp 41) (figure 18D). However the EVA catalogue gave the target sequence as FKQSSGGDPEIVTHSFNCGGE (aa 362-381 of gp120) (table 6). The EVA Program was informed about our discrepant result. They contacted Dr. Thiriart and found out that the target sequence that had been given by this mAb was an error.

The intensity and clarity of staining spots obtained with these four mAbs was quite different. This can be explained by the fact that mAbs EVA3012 (figure 18A), EVA329 (figure 18B) and EVA328 (figure 18D) were contained in tissue culture supernatant, while EVA3063 (figure 18C) was provided as highly purified IgG.

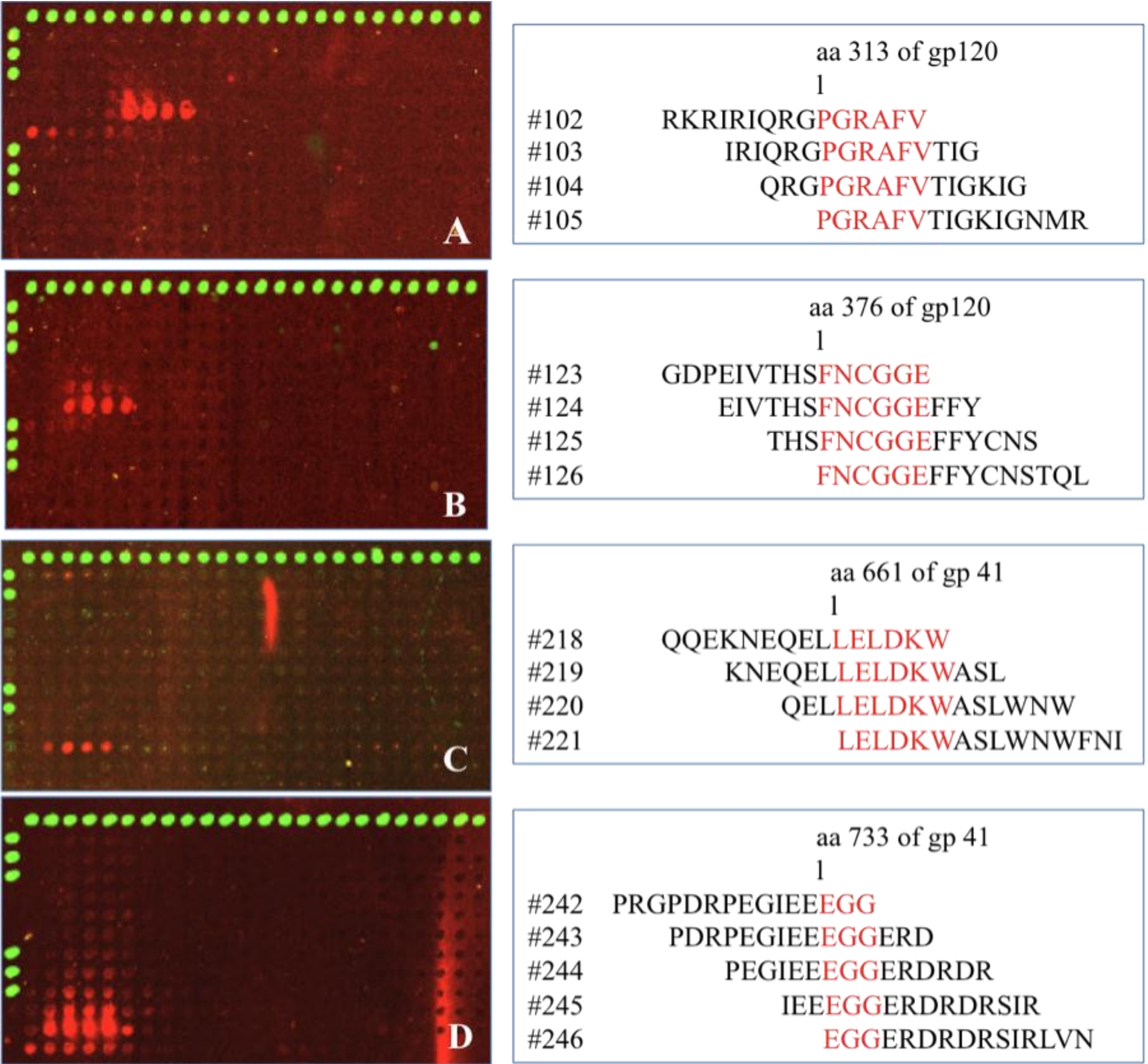


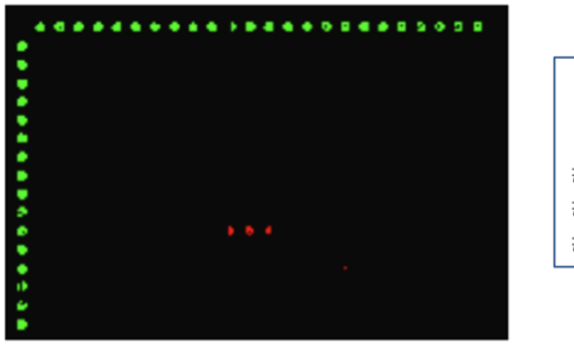
Figure 18: Results obtained with four mAbs on HIVenv chip. (A) EVA3012 mAb, (B) EVA329 mAb, (C) EVA3063 mAb, and (D) EVA328 mAb. Left is the one array field of the inspected HIVenv chip. Right are reactive overlapping peptides and the target sequence is shown in red.

3.2.2.2 Discrimination of HBV genotypes and serotypes with mAbs

According to their genome sequences, HBV isolates are classified into 8 genotypes (A-H). Each genotypes has a specific geographical distribution, e.g. in Europe mainly A and D. The most antigenic part of the HBV small-surface antigen (SHBsAg) is the antigenic loop (AGL). Variability in the AGL determines the nine HBsAg serotypes (Weber 2005; McMahon 2009; Kurbanov, Tanaka et al. 2010). The cross reactivity of the HB antibodies determines the broadness of the neutralization capacity. Our HBV chip contains the sequences of two genotypes found in Europe (A and D) and the nine HBV serotypes (Schaefer 2007). The targets sequences of these mAbs (2-11B1, HB1, HB3, and HB7) on different HBV genotypes or serotypes were determined.

3.2.2.2.1 Reactivity of 2-11B1 mAb

The 2-11B1 mAb was prepared by immunizing mice with HBsAg from HBV-carriers provided by Prof. Dr. Wolfram Gerlich, Institute of Medical Virology, University of Giessen. 2-11B1 mAb was screened with HBV scanning chips. As shown in figure 19, this mAb reacted with three peptides (202-204) leading to target sequence MQWNSTTFH (aa 1-9) of preS2 region of HBV genotype D. It did not react with genotype A. The only difference between both genotypes is at aa 7 where T found in genotype D is replaced by A in genotype A. This results target sequence was also found earlier by others with competitive ELISA using peptides containing either T or A (Sobotta, Sominskaya et al. 2000; Sominskaya, Paulij et al. 2002).



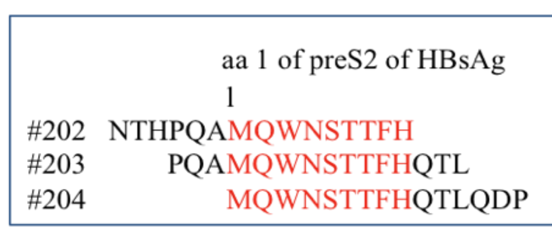


Figure 19: 2-11B1 reacting peptide on HBV microarray. Left, HBV scanning chip incubated with 2-11B1 mAb. Right is the recognition sequence.

3.2.2.2.2 Reactivity of HB mAbs

HB mAs (HB1, HB3, and HB7) were generated by immunizing mice with recombinant small hepatitis B surface antigen (SHBs, genotype D) produced in yeast. They were a gift from Prof. Dr. Wolfram Gerlich, Institute of Medical Virology, University of Giessen. These three mAbs were screened with the HBV chip. All of them detected 19 peptides on the HBV chip (figure 20 and table 7). The target sequence of this monoclonal was C(K/R)TCT(T/I) (aa 121-126 of SHBsAg). The second position of the recognition sequence can be either L or R and the last position either T or I. These mAbs react with all known HBV serotypes except serotype *ayw3*. This serotype contains M at aa position 125 (spot numbers 149-151 in table 7). These results exemplify the superiority of our chip technology, which detects antibodies against various genotypes or serotype in one step.

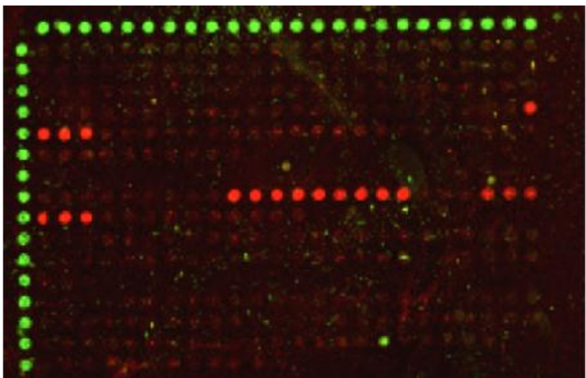


Figure 20: Screening HB1 mAb on HBVchip.

Table 7: HBV serotype-specific peptides and reactivity of their mAbs.

Spot number	aa sequence (121-126) of HBsAg of all serotypes	Serotype	Reactive mAbs
#096	GTTTTSTGPCKTCTT	*adw2*	All HB mAbs react with these spots
#097	TTSTGPCKTCTTPAQ		
#098	TGPCKTCTTPAQGNS		
#099	CKTCTTPAQGNSMFP		
#140	TSTGPCRTCTTPAQG	*ayr* *ayw1* *ayw2*	All HB mAbs react with these spots
#141	TTSTGPCRTCTTPAQ		
#142	TGPCRTCTTPAQGNS		
#143	TSTGPCRTCTTIAQG	*ayw4*	
#144	TTSTGPCRTCTTIAQ		
#145	TGPCRTCTTIAQGNS		
#146	TSTGPCKTCTIPAQG	*adr* *adrq*	
#147	TTSTGPCKTCTIPAQ		
#148	TGPCKTCTIPAQGNS		
#149	TSTGPCRTCMTTAQG	*ayw3*	No antibodies react with these spots
#150	TTSTGPCRTCMTTAQ		
#151	TGPCRTCMTTAQGNS		
#152	TSTGPCRTCTTLAQG	*ayw4*	All HB mAbs react with these spots
#153	TTSTGPCRTCTTLAQ		
#154	TGPCRTCTTLAQGNS		
#155	TSTGPCKTCTTLAQG	*adw4*	
#156	TTSTGPCKTCTTLAQ		
#157	TGPCKTCTTLAQGNS		

The C(K/R)TC motif is highly conserved among subtypes and mutants of HBsAg isolates (Qiu, Schroeder et al. 1996). The H166 is an mAb reactive with the C(K/R)TC motif. Diversity of aa surrounding the tetramer did not interfere with the reactivity with H166 mAb. In addition, the reactivity of H166 mAb was preserved upon alkylation of the synthetic peptide containing such a motif, however, was completely destroyed upon reductive alkylation of the peptide (Chen, Delbrook et al. 1996). These results suggest that H166 mAb detected the C(K/R)TC motif in its loop format with a disulfide bond holding the two cysteines together. In the case of HB mAbs, they detected with the linear form of C(K/R)TC motif because the SH group of the cysteine of peptides prepared by SPOT technology were protected by trityl (Fields and Noble 1990; Frank 1992). The difference in reactivity between these mAbs might be due to the fact that the H166 mAb was prepared with mice immunized with HBsAg particles from human plasma, while with HB mAbs, the immunogen was from recombinant SHBs particles produced in yeast. Thus the yeast expresses the SHBs in another format than the natural antigen.

3.2.3 Results obtained with mAbs (MA18/7 and EVA3047 mAbs) on scanning chips are reproduced and expanded with a random peptide library

4608_RPL is a library containing 4608 random 15-mere peptides. Here it was used to identify target sequences of mAbs. MAbs (MA18/7 against HBsAg and EVA 3047 against HIV gp120) with known target sequences were screened with HBV miniarray and HIVenv chips and thereafter with 4608_RPL. The aa sequences of peptides reactive on 4608_RPL were aligned to derive the binding motif of these mAbs. The importance of each aa within the binding motif could be analyzed by comparison of the respective peptide sequences.

3.2.3.1 Reactivity of MA18/7 mAb

MA18/7 was generated by immunizing mice with purified HBV particles (Heermann, Goldmann et al. 1984). It was reported to recognize DPAF (aa 31-34 of preS1 in genotype A) (Germaschewski and Murray 1995). This MA18/7 was screened with the HBV miniarray where it recognized DPAFGA (aa 31-36 of preS1 in genotype A) (figure 21). The additional two aa at the C-terminus are not part of the binding site of the mAb but rather belong to the three aa shift of each overlapping peptide on our HBV miniarray.

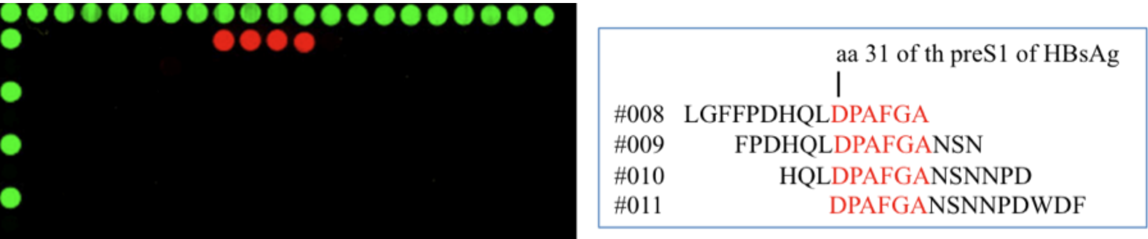


Figure 21: MA18/7 reacting peptide on HBV miniarray. Left is the screening of HBV miniarray with MA18/7. Right are reactive overlapping peptides and the target sequence is shown in red.

The MA18/7 was also screened with 4608_RPL (figure 22). It recognized 17 peptides, 11 strongly and 6 weakly (table 8). Alignment of the 11 strong responders revealed DPAF as the consensus sequence. The D at the first position was present in 7/11 strong responders. In the remaining 4 strong responders, D was exchanged with E (three times) or H. The Pro at the second position was found 3/11 strong responders and was replaced either by A, K, R, Q, or W (table 9). The A in the third position was found 6/11 and could be replaced either by G or C. In the fourth position, F detected 9/11, and could either be exchanged by W or Y. Germaschewski and Murray, as well as D´Mello and his colleagues, used a phage display library of $2*10^8$ or $2*10^9$ clones, respectively (Germaschewski and Murray 1995; D'Mello, Partidos et al. 1997). In an experimental by Germaschewski and Murray experiment, 30 peptides were identified; 6 of which did not align with DPAF (table 9). In the other 24 peptides, D and P were only allowed in both the first and the second positions respectively, while in the third position A was identified 13 times, G 9 times, and S, and V once. In the fourth position, F was present 22 times and Y twice. D´Mello and colleagues identified 20 reactive peptides. Only D and F were allowed in both the first and fourth positions, while the second and third were promiscuous (table 9). As described above, our experiment with the 4608_RPL and the results of the groups using the phage library derived the DPAF as a target sequence of the MA18/7 mAb.

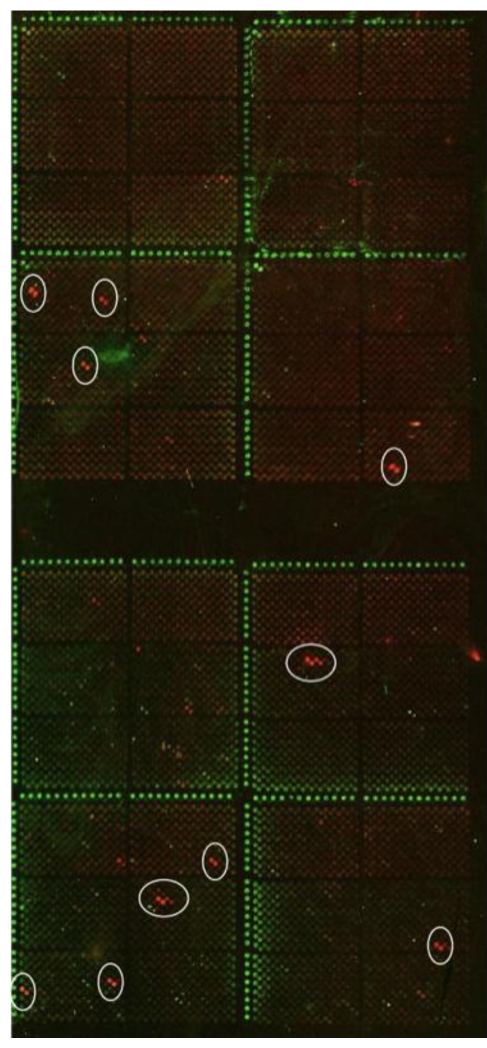


Figure 22: EVA3047 mAb screened with 4608_RPL. The strong responders are circled in white.

Table 9: The peptides reactive with the MA18/7 mAb. The detected motif is colored in red.

Nr.	Spot number	Sequence	Class
1	Q13D2	GRVALYTEPGFRVQY	S
2	Q13E10	SIQCHQHHKAFQSRY	S
3	Q17D8	VDQAFKTFQAERKHT	S
4	Q38G10	TYWDRGFQGWYGMIN	S
5	Q42C4	MASEFTQALDAAFFK	S
6	Q45D11	VMPYPETEPAFLDKC	S
7	Q45E1	SAYQIFMEMWSDKAF	S
8	Q24G4	TMQHDKCWQYWFLCS	S
9	Q31B7	CWFNCMKMDPGFKTI	S
10	Q31B8	LQLQGYMMHRVEWCY	S
11	Q44H9	SNFPDAAFNNQEGID	S
12	Q44H10	FGVACFNFIHWGEMD	W
13	Q48E7	QMWYVYCMDAGYRYR	W
14	Q10A11	DFPFNTYGKWNHSTI	W
15	Q22D5	MDEHNWVDQWMTQYM	W
16	Q11A12	DCFTIWPAFIKSGDQ	W
17	Q42C5	NRVRIAWRDRINAQN	W

S is strong responder and W is weak responder

Table 9: DPAF was identified as the target sequence of MA18/7 from the peptide profiles obtained in three independent experiments.

4608_ RPL

D (7)	P (3)	A (6)	F (9)
E (3)	K (3)	G (3)	Y
H	A (2)	C (2)	W
	R		
	Q		
	W		

Germaschewski and Murray 1995

D (24)	P (24)	A (13)	F (22)
		G (9)	Y (2)
		S	
		V	

D'Mello, et al. 1997

D (20)	P (12)	A (10)	F (20)
	R (6)	V (5)	
	S	G (4)	
	L	S	

An important technical issue is the target sequence of an mAb, which can be identified with 4608_RPL in around 6 hours, whereas preparation and screening of phage display libraries may take a month to perform. In addition, in phage display libraries, some DNA inserts are over-represented and other immunologically important ones may be missing. Therefore, the 4608_RPL is a simple and powerful tool to identify targets of mAbs.

To further consolidate these results, MA18/7 was reacted with 120 15-mere peptides in which each of the 4 aa of the binding motif is exchange by either of the 20 aa (figure 23). Only one aa substitution per peptide was allowed. As a result, D at the first position can only be replaced by E or H. The second position P is more promiscuous and less significant for the MA18/7 recognition. The third position can either be A, G or V and the forth F or L. A very similar result was obtained with 4608_RPL except for the third and fourth positions (table 9). D´Mello's results resemble ours for the second and third positions, while Germaschewski and Murray´s for the third position only. Thus the results obtained in three independent experiments are similar but not identical. This might be due to the fact that in the single residue substitution assay only one aa of the binding sequence was exchanged, while peptides of the 4608_RPL or the phage display libraries recognized by MA18/7 contained multiple aa exchanges.

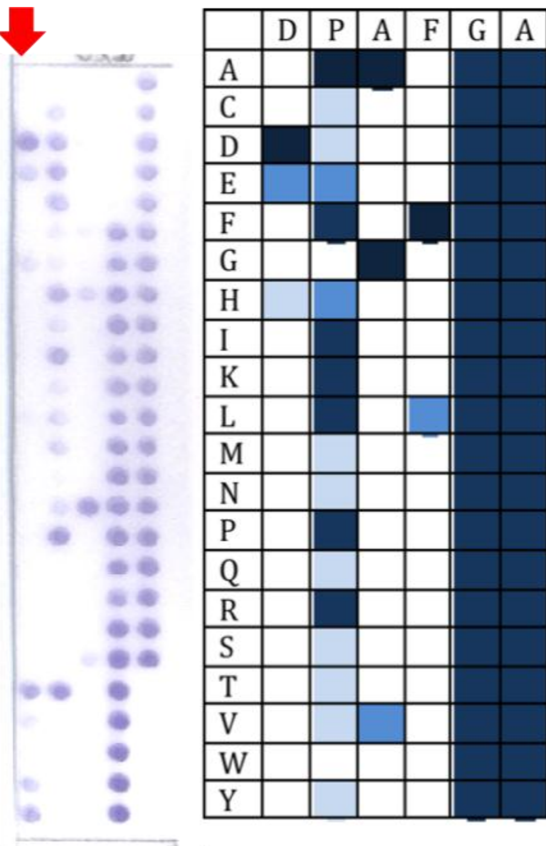


Figure 23: Single aa residue substitution assay identified the MA18/7 binding motif. Left, reactivity with the 15-mere spotted on a nitrocellulose sheet. The first position of the binding motif is indicated by a red arrow. Right, signal strength in quartiles (Dark blue strong reactivity and white no reactivity).

3.2.3.2 Reactivity of EVA3047 mAb

The EVA 3047 mAb from Dr J Laman was provided by the EU Programme EVA Center of AIDS Reagent, attached to NIBSC, UK. It was generated by immunizing mice with the gp120 HIV-1 peptide (IRIQRGPGRAFVTIGC). The reactive sequence of EVA3047 was found to be QRGP (aa 310-313 of HIV gp120) (Laman, Schellekens et al. 1992). It was screened with the HIVenv chip. The target sequence was identified as QRGPGR (aa 310-315 of HIVgp120) (figures 16 & 17). The additional two aa at the C-terminus in our sequence were explained above.

The EVA3047 was also screened with the 4608_RPL. Twenty-four binding peptides were identified, five as strong responders (figure 24 and table 10). Aligning the five strong responders revealed the QxGP as the target sequence of EVA3047. X was M, R, or V.

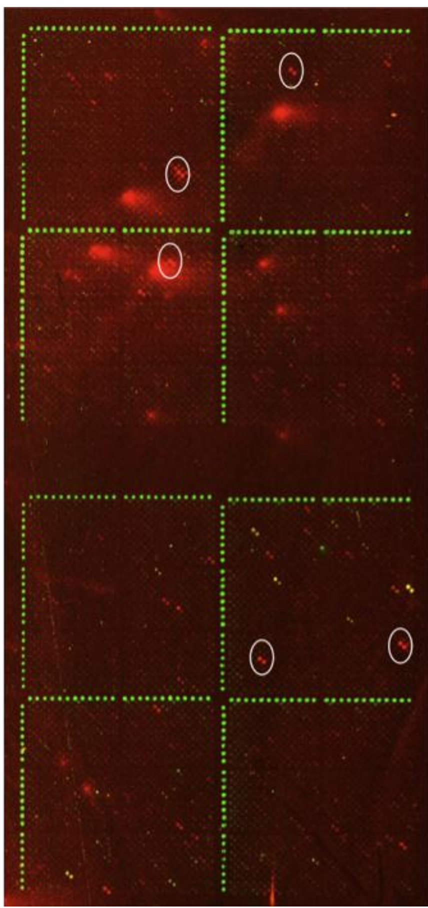


Figure 24: EVA3047 mAb screened with 4608_RPL. The strong responders are circled in white.

Table 10: The peptides reactive with the EVA3047 mAb.
The detected motif is colored in red.

Nr.	Spot number	Sequence	Class
1	Q10B8	KSPPMWYQMGPRMQE	S
2	Q3E9	LHQYEYMREQFKGWT	S
3	Q14D7	QDSWVQMQQSIWRLR	S
4	Q36B11	CLYNYLCWVDRQVGP	S
5	Q35D5	WGWMYPLEQDQRGPR	S
6	Q1F6	FNRQSWRQEQRKANY	W
7	Q48B11	EYTFPFRHQMVVFRW	W
8	Q1F11	NPQMHNKLVYNMHQY	W
9	Q4D9	FMVESHQFLYSHRRR	W
10	Q14H3	DRGQGRFKQMTYNIW	W
11	Q24H8	CECGQRILIRGQRQI	W
12	Q21A3	QQQFQLWFNMSACIR	W
13	Q24B10	FRGFATRMYYPSRAM	W
14	Q24C10	QMDQCRRRFTCQIWF	W
15	Q26H12	FTTWCQPGWGRFRHQ	W
16	Q30E6	GFHPRLMQQVGIMRS	W
17	Q27G6	RRKRHIFRRNRKKTC	W
18	Q27A11	QRIWNFSLSNFHQFE	W
19	Q27D11	LQLKHQFNMHTLQWS	W
20	Q28G4	RQFMDQGNWLGQQFQ	W
21	Q32C10	RSERESSVNYKTQTP	W
22	Q38A5	QHCPPDVPLDHQFVK	W
23	Q45H11	IEWFNHGPGMTLELA	W
24	Q42H8	FLPDLFPLHFHQFRH	W

S is strong responder and W is weak responder

As described above with the MA18/7 mAb, a peptide substitution assay was performed for QRGPGR sequence to define the relevance of each aa for the EVA3047 binding. R in the second, G in the third and fifth, and R in the last position are promiscuous and can be substituted with any of the other 19 aa (figure 25). Again the 4608_RPL was useful for identify the targets of four aa in length.

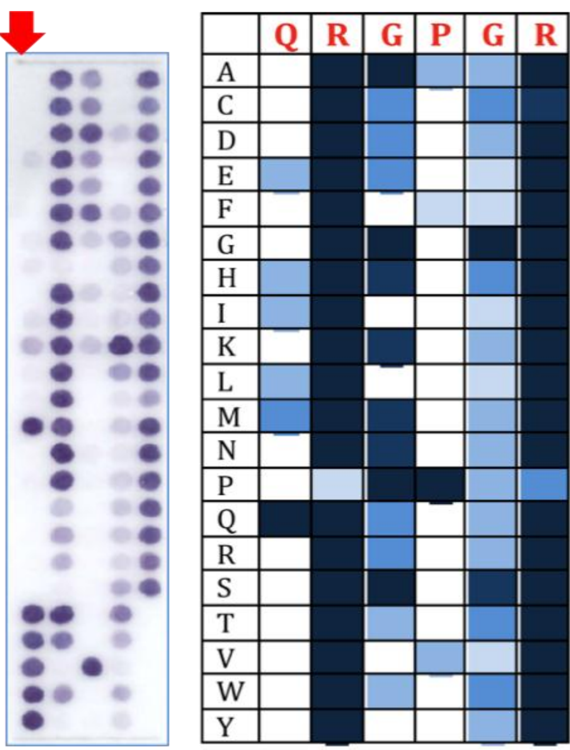


Figure 25: Single aa residue substitution assay identified the EVA 3047 binding motif. Left, reactivity with the 15-mere spotted on a nitrocellulose sheet. The first position of the binding motif is indicated by a red arrow. Right, signal strength in quartiles (Dark blue strong reactivity and white no reactivity).

3.2.4 No reactive peptide found on the scanning chips with HBV and HIV mAbs except for 4608_RPL

The paratopes on an antibody binds to the epitope of an antigen. Depending on the tertiary structure of an antigen, an epitope can either be continuous (linear) or discontinuous (conformational) (figure 26). A continuous epitope comprises a group of consecutive aa representing the primary sequence at a specific site. The mAbs MA18/7 and EVA3047 tested on 4608_RPL in figure 22 and 24 recognize continuous epitopes. Continuous epitopes withstand denaturation of the antigen. A discontinuous epitope comprises two or more groups of residues located close to each other in the tertiary structure. Denaturation destroys the reactivity of the discontinuous epitopes (Reineke 2004). Epitopes may also contain modified residues (e.g. glycosylation, phosphorylation) (Lisowska 2002). Here are attempts to identify targets of three mAbs C20/02, and EVA332 recognize discontinuous epitopes while Q19/10 recognizes modified residues.

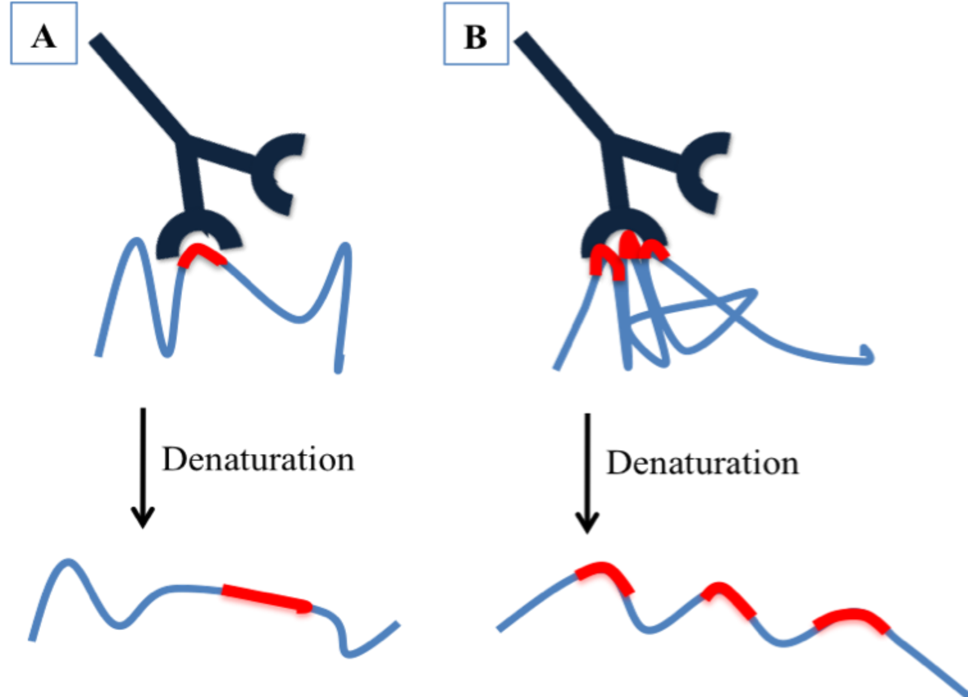


Figure 26: Antigen antibody interaction. (A) Continuous epitope. (B) Discontinuous epitope.

3.2.4.1 Reactivity of C20/02 mAb

C20/02 mAb (provided by Prof. Dr. Wolfram Gerlich, Institute of Medical Virology, University of Giessen) was generated by immunizing mice with subviral particles from HBV derived from a chronic carrier. It reacted neither with western blot (Wolfram Gerlich, personal communication) nor with the HBV scanning chip. Thus it might recognize a discontinuous epitope or epitope of modified residues. C20/02 recognized 72 peptides on 4608_RPL. Fifteen were strong responders (table 11 and figure 27) while 57 reacted weakly (table 12). The alignment of the 15 strong responder sequences using the MUSCLE program available from EBI revealed YKP+F+G++GWG+N-M (Figure 28A) as a consensus sequence. This consensus sequence was compared with aa 100-180 of SHBsAg genotype D. The consensus sequence aligned with three parts of the SHBsAg: aa 100, aa 141-145, and aa 165 (figure 28B).

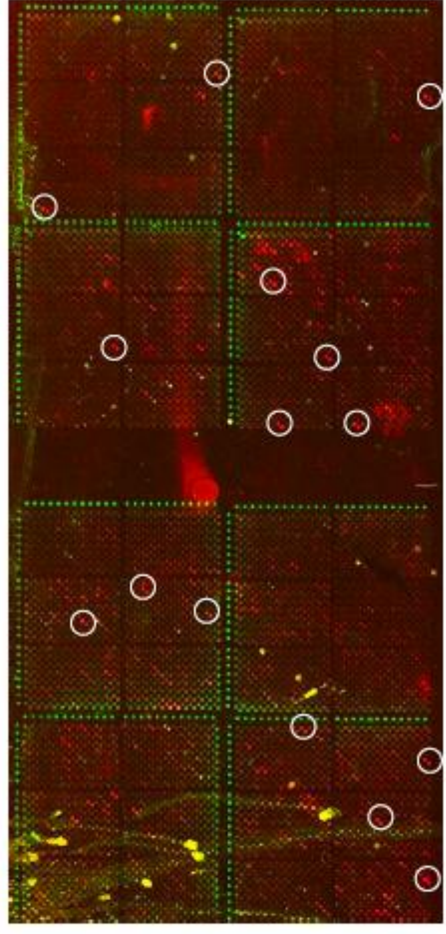


Figure 27: C20/02 mAb screened with 4608_RPL. The strong responders are circled in white.

Table 11: The peptides strongly reactive with C20/02 mAb.

Spot number	Sequence
Q9H3	GPDQCVGWMGGMQPW
Q2H12	PKAITGEDFWDATRM
Q17G12	ARGEGWMIPLKFRFI
Q29F8	NYDRLYRREPGTGGN
Q30B3	NWKFRFCYMACLFRI
Q30E11	GFKPCYYFRDMAEFD
Q8B12	ALTCAGNIPNKLFNM
Q15G5	FFKSVCNGPMAVGWA
Q19H12	INPPKFFQRNEWMWH
Q23H6	YAPMCVGIRGITPKV
Q24H3	GGIFRGKPVYQEYHM
Q39A9	SQTIVDKFRGYMRNF
Q40E12	AEPMVNPFAFHAFRP
Q44D6	FYAPPLIGAQDWYYN
Q48C12	GYVLPLFYGKQHMGP

Table 12: The peptides weakly reactive with C20/02 mAb.

Spot number	Sequence	Spot number	Sequence
Q6B10	MTPWNCWCIFNWLQF	Q12F4	HCVPRAFWNATMYWN
Q13D11	FISRKVMNNVGWYIN	Q15B7	WNRRHKPQYAFFQGL
Q13H9	KFPVGEYVLWSFRVK	Q15C5	YEHNFWCFRTYFPNV
Q18B4	IPDKWKAGMKWRDKK	Q15F1	FCFADTLFRKHRQAN
Q18G10	FLEFCWCMAFRWANV	Q15F6	THGLVFNRGEWSIWD
Q22D3	GRSISLMFPFWMQMI	Q15H7	DENFFKPWKRIVFKR
Q26H9	QFKGFPGRADDFLFF	Q15H11	SMRWPVHTYKPTMFT
Q33D11	VRNSRFPRCKFRSPA	Q16E7	NQMVLNSCPSYYPCG
Q33F3	WWRRCRFNNHLGMVF	Q20A8	VKGCIWQFTKPMFFL
Q34E11	FMMAYQWPFFNFRAD	Q23A12	RWWTGAWAKFNPRIS
Q34E6	GSVEPKFREQEKLCW	Q27G2	RAPWMFFSPYLGITN
Q34C4	MKCMYRKALDYPGYM	Q27G5	KVHTICAMLFRLRPW
Q37E6	GSHIKFFFRGYGHWP	Q27G8	SIGNGRIPINFGIDW
Q38A4	YLVTKTYFWRKYRTF	Q31D3	PCCPCFWLQMFMSPP
Q38B11	TWQFRIAREWFMPCG	Q39B11	SQFTFCAKIREGGFF
Q38C9	WMKVVNQEFRIFNMN	Q39C2	NKMLWYGFMNQPARQ
Q41A12	LNEYARMWFNAQPGY	Q39F4	RPYRSYHRRFMGWMD
Q41B9	SAMCWNSAVRTIWRA	Q43A10	TNICVFRKTKKFMTA
Q41D7	YMGTPWFFAAPFMRR	Q43C10	MSGNTWSPKNHMFNI
Q42C2	EALKNLYFNLSAKMS	Q43D2	HSHTRSLYDPFSDGY
Q42F4	YVEHVAYVELNRRYL	Q43D12	ELQCMWRVSHGMQWF
Q45E3	RNIFRCRYRQILWGF	Q43F7	YAQCVGYMYVFRWCT
Q45G2	IMACILPNFYYSGRF	Q43F11	FFRAGSLMEWLNEFI
Q46A2	YSITGFEEQTVHTNW	Q43G9	TNDENPYIFWSVFRR
Q46G3	LVVRFCINKVIRFIT	Q48D1	PFGWWMMGYLYDATP
Q3G6	PQPFIHVNMLFRYRV	Q48E7	QMWYVYCMDAGYRYR
Q4E6	TWPQGWKFKNLGNGE	Q48G6	KFRYTIGNTYGNPCH
Q4G5	RWRDSSLGYKFRLAF	Q48G7	HGGQCPYGALYGSCP
Q8G8	YGVWDCFSTPCVGGL		

Several motifs could be extracted from the peptides reacting with C20/02. (1) KxxxG/R motif was found in 3 of the strong responders and in 7 of the weak responders (violet in tables 11 & 12). This motif might represent aa 141-145 of SHBsAg. (2) 6/15 strong responders and 2/57 week responders contained PxxxG/R motif (red in tables 11 & 12). (3) 9 peptides contained WxF motif (blue in tables 11 & 12). This motif might represent aa 156-158 of SHBsAg. (4) Spot number Q13H9 (orange in table 12) contained the motifs 1 and 3 of the consensus sequence KxxxGxxxxWxF. (5) A motif of 3 residues encompassing G at the first position, any of the 20 aa at the second position, and then a stitch of aliphatic aa was found in 10 reactive peptides (green in tables 11 & 12). This

motif might represent aa 102-104 of SHBsAg. (6) The YR motif was found in 8 peptides, while the closely related WR motif was seen in 6 (brown in tables 11 & 12).

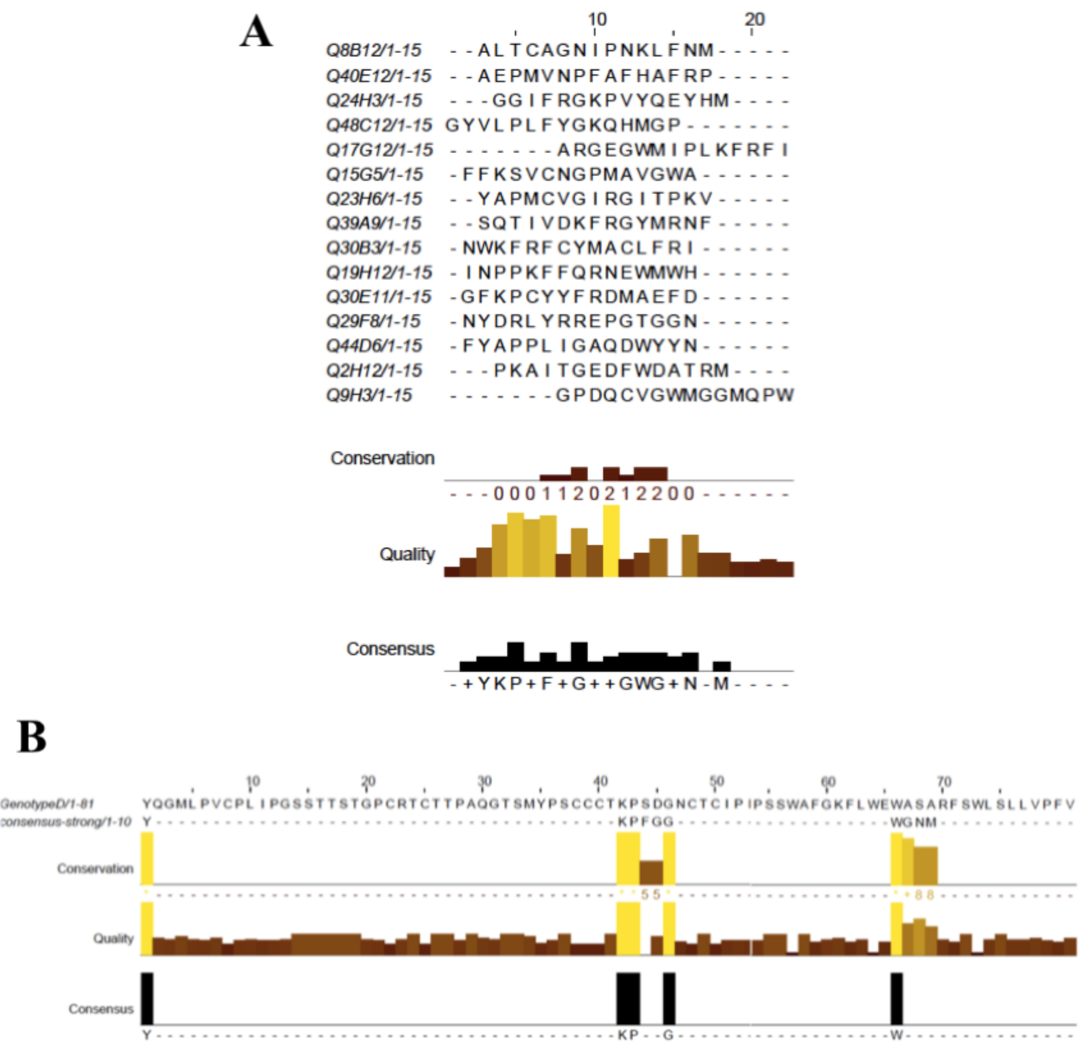


Figure 28: Alignment of the C20/02 strongly reactive peptides derived from 4608_RPL. (A) Alignment of the 15 strong responders. (B) Alignment of aa 100-180 of SHBsAg (upper row) with C20/02 strong-responders-consensus sequence (lower row). This alignment was done using the MUSCLE program. The numbers above the sequences in the first row represent the numbering in the MUSCLE program not the position within the SHBsAg. The program placed the consensus sequences in three parts within the SHBsAg (aa 100, aa 141-145, and aa 165 of SHBsAg).

In an independent experiment performed by Prof. Dr. Camille Sureau, Laboratoire de Virologie Moléculaire, Institut National de la Transfusion Sanguine, Paris, a panel of HBV subviral particles bearing the 3 envelope proteins **L**-, **M**- and **S**HBsAg (LMS) was constructed. Each LMS mutant bears an amino acid substitution at one position of HBV

AGL (residues 101 to 172), S for C or A for non-C. A couple of substitutions (grey in the histograms, figure 29) could not be produced. For C20/02 epitope mapping the antibody was fixed to wells in a Maxisorp ELISA plate and reacted with LMS particles. Bound particles were detected by anti-preS2 antibodies.

The LMS binding profile of C20/2 is shown in figure 29. Binding of C20/02 was affected by many residues. Some substitutions seemed to improve recognition e.g. T118A, T123A, M125A, T127A, Y134A, S136A, and T149A. But substitutions of all C by S were detrimental. This was expected because disulfide bonds between C keep the HBsAg tertiary structure. Moreover, the C20/02 reactivity was lost with the following substitutions G119A, P120A, T140A, K141A, D144A, and G145A. In addition, LMS particles with substitutions in the C terminus of SHBsAg (some single aa substitutions and deletions between residues 172 and 226) had no effect on C20/02 binding (unpublished data).

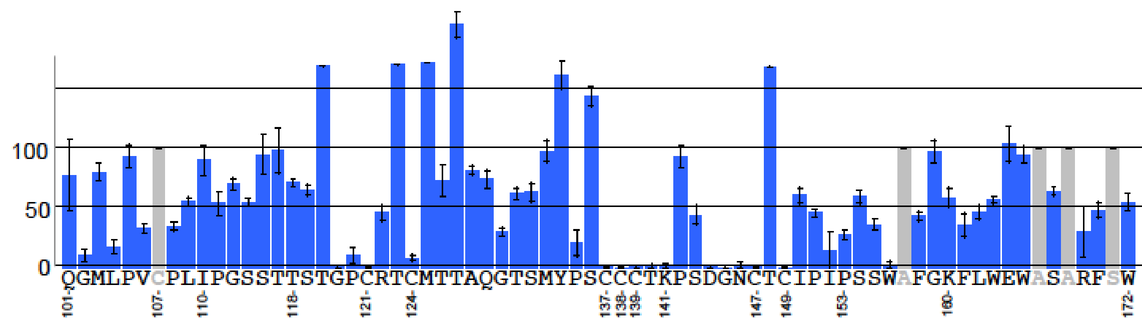


Figure 29: Reactivity of C20/02 with LMS. LMS are different HBV subviral-particles, each containing an amino acid substitution in the AGL. The gray columns indicate non-permissible mutant. The effect of each substitution on the recognition of C20/02 was tested in ELISA. C20/02 did not detect LMS having substitutions of cysteine with serine in addition, G119A, P120A, T140A, K141A, D144A, G145A, and N146A (unpublished data).

Thus both our 4608_RPL and Dr. Sureau's LMS experiments showed that the binding site of the C20/02 might include three parts within AGL, the N-terminal, in the middle part, and the C-terminal sequences. Since the HBsAg 3D structure has not been determined (Ren, Tsubota et al. 2006), our results could be of great help in constructing a 3D model of HBsAg.

3.2.4.2 Reactivity of Q19/10 mAb

Q19/10 (provided by Prof. Dr. Wolfram Gerlich, Institute of Medical Virology, University of Giessen) was generated by immunizing mice with purified HBsAg/*adw* subviral particles from a chronic HBV carrier. Q19/10 target sequence is the N terminus of the preS2 of the middle hepatitis B surface antigen (MHBsAg), i.e. the N-glycosylated aa 1-6 of preS2 (MQWNST) of MHBsAg (Heermann, Waldeck et al. 1988; Glebe, Aliakbari et al. 2003). When screened on an HBV chip. Q19/11 did not recognize peptides. This was expected because this antibody is glycan-dependent. Interestingly, it recognizes 20 peptides within the 4608_RL (figure 30 and table 13). One of these peptides (spot number: Q41B9) contains MCWNSA, which shared 4 residues with aa 1-6 of preS2 of MHBsAg (expectation value 0.019) (red in table 13).

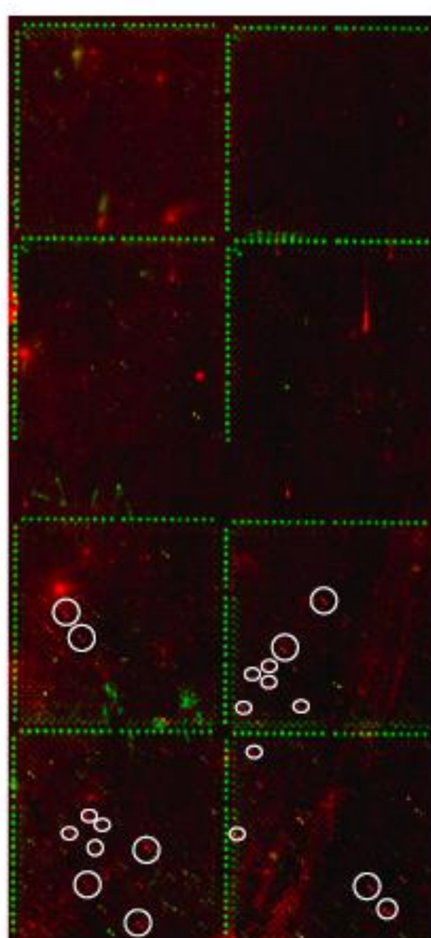


Figure 30: Q19/10 mAb screened with 4608_RPL. The reactive peptides are circled in white.

Table 13: The peptides reactive with the Q19/10 mAb.

Nr.	Spot number	Sequence
1	Q29C6	ARAESPRNFWRRCCL
2	Q29F8	NYDRLYRREPGTGGN
3	Q41B9	SAMCWNSAVRTIWRA
4	Q41C11	RCRFPYGMMANKWGL
5	Q41D7	YMGTPWFFAAPFMRR
6	Q41F10	HRWYIPHIFGRFDGK
7	Q45B9	PWINCVVCKAFHHNP
8	Q46G3	LVVRFCINKVIRFIT
9	Q42F4	YVEHVAYVELNRRYL
10	Q31A12	RSWYRKQTINNMNIT
11	Q31G7	GRCGVWYGNKCYARW
12	Q35A5	CGVGFSRSTIMNYRG
13	Q35B3	RFRWIHPHRHMFHGN
14	Q35C5	CLNWYWMPSVIRQRW
15	Q35F1	DCTVTHGHGTLCYRF
16	Q35F9	YFGKITRCVNRHYQK
17	Q39B3	IHYSSCLICVIMKMA
18	Q43D1	ISPTTKGQYAEGQSV
19	Q48B5	CRYFQYYNNCLPPIM
20	Q48E7	QMWYVYCMDAGYRYR

The reactivity of Q19/10 mAb with peptides of the 4608_RPL, which lack the glycans, might indicate that the Q19/10 did not detect the glycan itself. But the glycan rather helps to present the epitopes in a way compatible with binding and this structure effect can be mimicked by an appropriate aa sequence. Several studies showed N-linked glycosylation influences on the protein folding (figure 31). A study presented the importance of glycosylation in determining peptide antigen structure and function. Synthesized glycosylated and phosphorylated versions of the two peptides were compared with the native peptide using CD and FT-IR spectroscopy. The data showed that incorporation of glycan into peptides resulted, with a high probability, in a type I β-turn formation (Otvos, Thurin et al. 1991). Huang X and his colleagues prepared glycosylated analogues of the principal neutralizing determinant of HIV gp120 and studied their conformations by NMR and circular dichroism spectroscopies. Moreover, they studied the binding affinity of 0.5β mAb with these particles. The data showed that glycosylation of gp120 peptides could affect their conformations as well as their interactions with antibodies (Huang, Barchi et al. 1997).

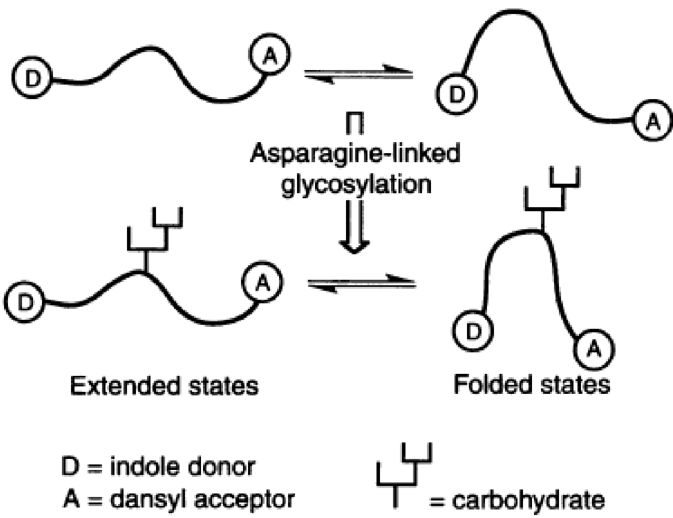


Figure 31: Influence of N-glycosylation on peptide folding (Imperiali and Rickert 1995).

Another observation made with the 20 positive peptides was that the YR motif presented 6 times (expectation value 0.7) (blue in table 13). Currently, there is no explanation for the reactivity of Q19/10 with such a motif.

3.2.4.3 Reactivity of EVA332 mAb

The EVA 332 mAb from Drs. C. Thiriart and C. Bruck was provided by the EU Programme EVA Center of AIDS Reagent, attached to NIBSC, UK. EVA332 was generated by immunizing mice with recombinant HIV-1 gp160 from yeast. The reactive sequence of EVA332 was found to be aa 101-120 (VEQMHEDIISLWDQSLKPCV) (Thiriart, Francotte et al. 1989; Moore, Sattentau et al. 1994). It recognized no peptides when screened with the HIVenv chip.

Screening with the 4608_RPL revealed 178 reactive peptides (figure 32, table **S**5). Feeding their sequences into the motif recognition program "Meme" lead to [VT]E[HQR][MC][QN]C[KG]G as a suggested target for EVA332. The first four positions of the motif contain aa 101 - 104 of gp120 (VEQM). This sequence is equivalent to the first four positions of the targets identified earlier. The last four positions of the motif contain the string 377 - 380 of gp120 (NCGG). This might indicate that EVA332 recognizes a discontinuous epitope composed of the two strings VEQM and NCGG. The reactivity of the EVA332 in the competition assay of Thiriart et al., 1989 might be due to specific experimental conditions, e.g. concentrations of mAb, and/or peptides sufficient for mAb binding with low affinity.

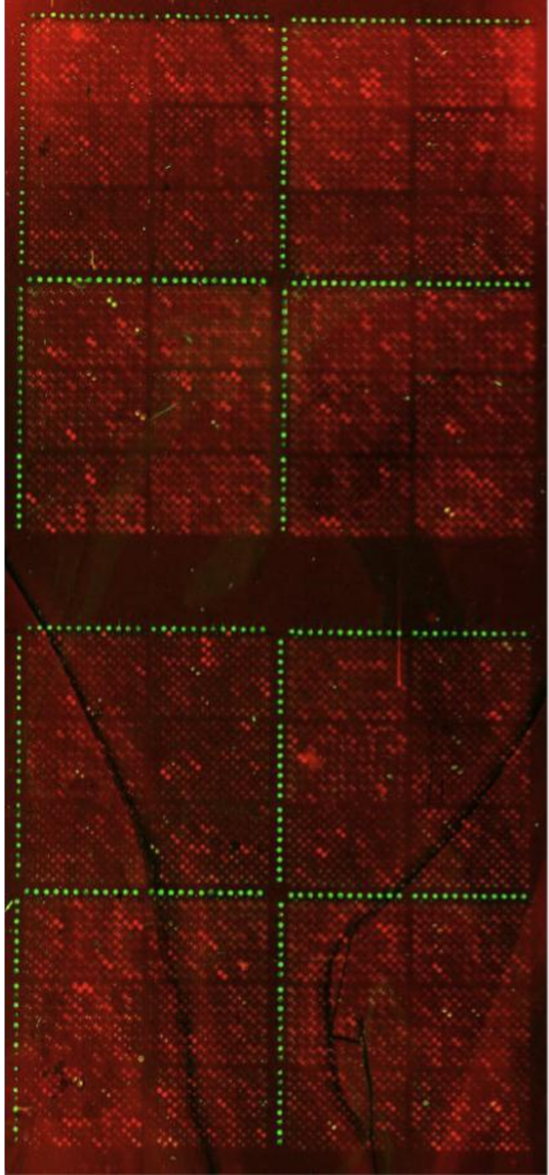


Figure 32: EVA332 mAb screened with 4608_RPL.

3.2.5 No unambiguous results obtained with the random peptide library despite clear identification of target sequence on the scanning chip

The target sequences of 6 mAbs (2.11B1, HB, ARP301, ARP3051, EVA3046, EVA3048) were identified using the scanning chips (table 14). Screening them with the 4608_RPL derived a number of reacting spots (table 14) but the targets could not be determined. However, the targets of mAbs MA18/7 and EVA3047 could be identified with the 4608_RPL. This might be explained by the short target size of 4 aa of MA18/7 and EVA3047, while targets of HB1 and EVA3048 comprised six residues, of 2.11B1, ARP301, and ARP3051 nine, and of EVA3046 twelve residues. Moreover, our random

library contains 4608 15-mere peptides. To detect motifs of 6, 9, or 12 aa, a larger random peptide library might be needed.

Table 14: Target sequence and the recognized peptide of six mAbs.

Mab	Target sequence	Recognized peptides on the 4608_RPL
HB1	aa 121-126 of SHBsAg (CKTCTT)	45 (figure **S**1 & table **S**6)
EVA3048 (IIIB-V3-21)	aa 295-300 of HIV gp120 (NCTRPN)	12 strong responders only (figure **S**2 & table **S**7)
2-11B1	aa 1-11 of preS2 of HBsAg (MQWNSTTFH)	31 (figure **S**3 & table **S**8)
ARP301(221)	aa 475-482 of HIV gp120 (MRDNWRSEL)	62 (figure **S**4 & table **S**9)
ARP3051 (SR3 (4D7/4))	aa 370- 378 of HIV gp120 (EIVTHSFNC)	23 strong responder only (figure **S**5 & table **S**10)
EVA3046 (IIIB-V3-01)	aa 319-330 of HIV gp120 (TIGKIGNMRQAH)	7 (figure **S**6 & table **S**11)

3.2.6 Synopsis and general conclusions

Use of the scanning chips (HIVenv and HBV) is a simple, rapid (6 hours) and reliable method to detect linear targets of mAbs. Targets of all mAbs used in this study focusing on linear sequence were identified using the scanning chips. The 4608_RPL contained targets of antibodies recognizing different types of epitopes; continuous 4 aa in length, discontinuous, and those with modified residues. However, the 4608_RPL could not identify sequences of mAbs detecting continuous epitopes of 6, 9, or 12 aa. Nevertheless, mimics of targets of these mAbs were detected. The 4608_RPL is the starting point towards developing a larger library that could serve as a universal tool to identify antibody targets (one chip serves all). There are several programs available online to identify mAbs targets. Some of them were successful with one mAb but not with others. Consequently, a special program that extracts the target-motifs from 4608_RPLbinding peptides is needed.

3.3 Reactivity of human sera to the HBV chips

HBV epiopes recognized by sera from HBV-vaccinated or -infected humans were identified with HBV microarray and miniarray.

3.3.1 Sera from HBV-vaccinated individuals

Two types of HBV vaccine are currently on the market. (1) Engerix-B (yeast-derived vaccine), the most widely used HBV vaccine contains only the SHBsAg produced in *Hansenula polymorpha*. (2) Bio-Hep-B (mammalian-derived vaccine), less widely used, contains the complete HBsAg comprising preS1, preS2 and SHBsAg produced in Chinese hamster ovary cells (Shouval 2003; Zhou, Wu et al. 2009). Both vaccines protect against HBV infection.

3.3.1.1 Epitopes detected by Engerix-B-vaccinated individuals

Forty-eight serum samples (provided by Dr. Angela Uy, UMG, Göttingen, and Prof. Dr. Wolfram Gerlich, University of Giessen) from healthy individuals vaccinated with Engerix-B were screened with HBV miniarray. 23/48 sera detected the CKTCTT motif (121-126 of SHBsAg, Ep#11 in table 17) on the HBV miniarray (figure 33A). Mouse sera (gift from Prof. Wolfram Gerlich, University of Giessen) obtained after vaccination with SHBs particles produced in yeast (like Engerix-B) also recognized the CKTCTT motif. Moreover, Folgori et.al., identified the same motif in a phage display library with two sera from immunized individuals (Folgori, Tafi et al. 1994). Since only about 50% of Engerix-B-vaccinated individuals had made antibodies against the CKTCTT motif, the high titers of all sera against HBsAg is best explained by additional antibodies reactive to discontinuous HBsAg epitopes. The presence of antibodies against glycosylated epitopes was excluded because the Engerix-B vaccine contains unglycosylated SHBsAg (Valenzuela, Medina et al. 1982).

Other studies detected reactivity of serum samples from individuals vaccinated with HBV-yeast-derived vaccine (like Engerix-B) to both the cyclic and linear forms of peptides corresponding to aa 139-147 of SHBsAg (Brown, Howard et al. 1984; Brown, Stanley et al. 1986; Emini, Ellis et al. 1986). The affinity of antibodies for the cyclical form of the peptide was higher than for the linear form. However, the linear of this peptide presented in the HBV microarray chip used in this study did not produce a signal

from the 48 human sera examined. These conflicting results may be explained by a difference in the detection assays, while in the studies mentioned above, the peptides were presented to the antibodies in high concentrations and in liquid. The antigen is fixed onto glass slides in the microarray chip. Surprisingly, aa 139-147 of SHBsAg was a part of the discontinuous epitope recognized by the C20/02 mAb (section 3.2.4.1). It might be that sera from vaccinees are able to detect the same discontinuous epitope as the C20/02 mAb.

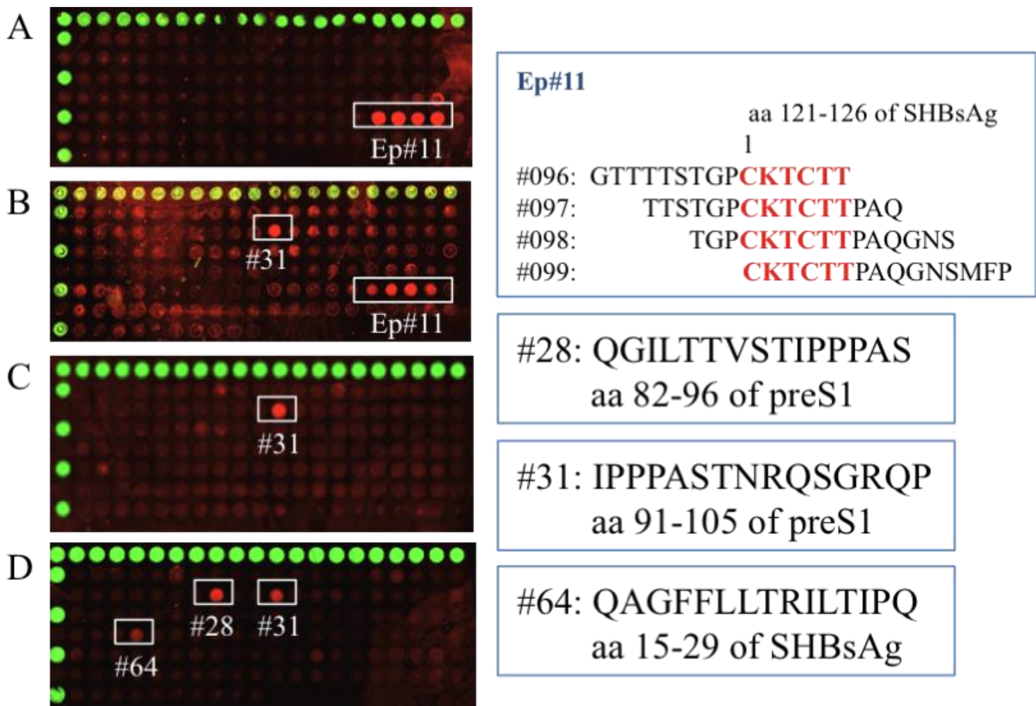


Figure 33: Serum reactivity on the HBV miniarray of 4 Engerix-B vaccinees.

3/48 sera recognized additional peptides: #28, #31, and #64 (figure 33B, C & D). Interestingly, peptides #28 and #31 are located in preS1 sequence. However, Engerix-B vaccine contains only the SHBsAg. Thus these individual were either infected with HBV or exposed to other pathogens with HBV-homologous sequences. Surprisingly, the sera reacted only with a single spot, while mAbs tested in this study reacted with at least 2 spots.

3.3.1.2 Epitopes detected by Bio-Hep-B-vaccinated individuals

Serum samples from 6 HBV-infected patients vaccinated with the Bio-Hep-B vaccine (not yet approved in Germany) were provided by Prof. Dr. Michael Roggendorf, Institute of Virology, University Clinic Essen. From each patient, pre- and post-vaccination sera were examined. The first individual recognized the same epitopes in both samples; individuals 2-5 did not detect any epitope, while patient number 6 had antibodies to one epitope prior to vaccination (table 15). Interestingly, this reactivity was lost after vaccination.

Table 15: Screening sera from Bio-Hep-B-vaccinated individuals with HBV chips.

Individual No.	Recognized epitopes	
	Pre-vaccination	**Post-vaccination**
1	46-KVGAGAFGL-54 of preS1 26-SVRDLLDNASALYR-39 of HBcAg	46-KVGAGAFGL-54 of preS1 26-SVRDLLDNASALYR-39 of HBcAg
2	--	--
3	--	--
4	--	--
5	--	--
6	184- VVRRRDRGRSPR-195 of HBcAg	--

Hellstrom et. al., examined the reactivity of 28 healthy newborns vaccinated with Bio-Hep-B vaccine with three synthetic peptides representing aa 21-32, aa 32-47 and aa 94-117 of the prS1. (Hellstrom, Madalinski et al. 2009). Fifty precent of the sera detected aa 21-32, 54% aa 32-47 and 43% aa 94-117. Interestingly, with sera available for this study, these three epitopes were never detected, probably due to the fact that they were derived from HBV-infected individuals producing an excess of HB subviral particles (Chai, Chang et al. 2008; Patient, Hourioux et al. 2009). Also the HBsAg discontinuous epitopes seemed to play an important role in protection (Chen, Delbrook et al. 1996; Germaschewski and Murray 1996).

3.3.2 Sera from HBV-infected patients (inf-Pa) in the recovery phase

72 inf-Pa sera (provided by Dr. Angela Uy, UMG, Göttingen) had antiHBs titer ranging from 175 to > 1000 iu/ml (AXSYM, Abbott). Independence from the antiHBs titer, 14/72 sera reacted with peptides on the HBV microarray (table 16). It could will be that the 58/72 non-reactive sera contained only antibodies to SHBsAg discontinuous epitope (Park, Cho et al. 2000).

Serum number HBV_#01 in table 11 detected 3 epitopes (Ep#10, Ep#11, and Ep#16); in addition, it reacts with all HBV serotypes (figure 34A). Serum HBV_#14 also reacted with Ep#11 but not all HBV serotypes (figure 34B). These two HBV-infected patients were vaccinated with the HBV-yeast-derived vaccine (Engerix-B) as a therapeutic trial. Ep#11 was also identified with sera from individuals vaccinated with the same vaccine as a preventive measure (see section 3.3.1.1). With the limited number of serum samples employed in this study, Ep#11 was only detected with sera from individuals vaccinated with Engerix-B but not with sera from infected but unvaccinated individuals. However the number of samples was too low to draw further conclusions.

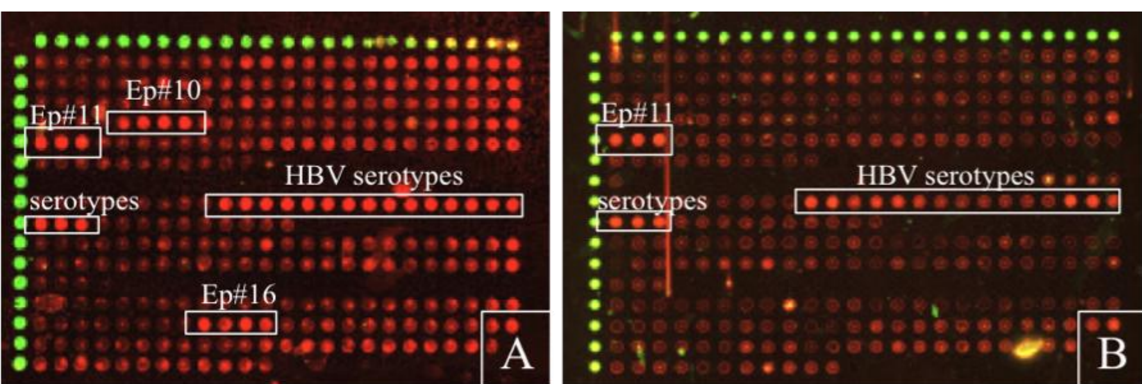


Figure 34: Reactivity of HBV_#01 (A) and HBV_#14 (B) on HBV chip. The epitope numbers are shown above the reacting peptides.

Serum HBV_#02 recognized 4 other epitopes (Ep#02, Ep#07, Ep#14, and Ep#15) (figure 35). Many sera recognized epitopes of either genotypes A or D, e.g. Ep#1 and Ep#3 (table 17). Others detected epitopes from both genotypes, e.g. Ep#02, and Ep#04 (table 17). Overall, 14/72 reactive inf-Pa identified 17 epitopes (Table 17, and figure 34 & 35). 10 epitopes (Ep#1 through Ep#9 plus Ep#11) have been identified earlier (Alberti, Cavalletto et al. 1990; Folgori, Tafi et al. 1994; Germaschewski and Murray 1996; Park,

Cho et al. 2000; Zhang, Wan et al. 2001). However, the remained 7 epitopes (Ep#10, and Ep#12- Ep#17) identified with the HBV microarray chip were detected for the first time.

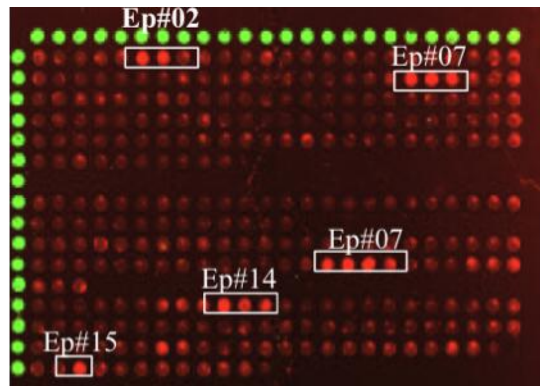


Figure 35: Reactivity of sample number HBV_#02 on HBV chip. The epitope numbers are shown above the reacting peptides.

Table 16: HBsAg epitopes reactive with sera from HBV-infected patients in the recovery phase.

No.	Code	Anti-HBs titer IU/ml	Anti-HBc	The identified epitope	Figure
HBV_#01	7701/07	>1000	+	Ep#10, Ep#11, Ep#16	34A
HBV_#02	10168/07	>1000	+	Ep#02, Ep#07, Ep#14, Ep#15	35
HBV_#03	10658/07	>1000	+	Ep#02, Ep#04, Ep#07	S7
HBV_#04	9912/07	323	+	Ep#07, Ep#14, Ep#15	S8
HBV_#05	10748/07	265	+	Ep#03, Ep#09	S9
HBV_#06	9922/07	175	+	Ep#09, Ep#12	S10
HBV_#07	10282/07	>1000	+	Ep#07, Ep#09	S11
HBV_#08	10248/07	>1000	+	Ep#02, Ep#04, Ep#07	S12
HBV_#09	10283/07	>1000	+	Ep#06, Ep#13	S13
HBV_#10	8927/07	>1000	+	Ep#02	S14
HBV_#11	8698/07	809	+	Ep #08, Ep#09, Ep#17	S15
HBV_#12	12071/07	1004	+	Ep#01, Ep#09	S16
HBV_#13	5097/07	1472	+	Ep#02, Ep#05	S17
HBV_#14	60/08	>1000	+	Ep#11	34B

Table 17: Sequence and position of the epitopes recognized by sera of HBV-infected patients in the recovery phase.

Epitope Number	Sequence recognized	Position	Number of sera detecting the epitope
Ep#01	GTNLSV	aa 2-7 of preS1, genotype A	1
Ep#02	LGFFPD PLGFFP	aa 11-16 of preS1, genotype A aa 10-16 of preS1, genotype D	5
Ep#03	NSNNPD	aa 26-31 of preS1, genotype A	1
Ep#04	PHGGVLGWSPQA PPHGGLLGW	aa 59-70 of preS1, genotype A aa 58-66 of preS1, genotype D	2
Ep#05	PTPLSP	aa 94-99 of preS1, genotype D	1
Ep#06	PQAMQWNST	aa 106-108 of preS1, genotype D + aa 1-6 of preS2, genotype D	1
Ep#07	DPKVRGLYF QDPRVRGLY	aa 13-21 of preS2, genotype A aa 12-20 of preS2, genotype D	5
Ep#08	TVNPVP	aa 31-36 of preS2, genotype D	1
Ep#09	PISSIFSRIGD HISSIS ARTGDPVTNMEN	aa 39-49 of preS2, genotype D aa 39-44 of preS2, genotype A aa 45-53 of preS2, genotype A + aa 1-3 of SHBsAg, genotype A	5
Ep#10	SCPPIC	aa 63-68 of SHBsAg, genotype A	1
Ep#11	CKTCTT	aa 121-126 of SHBsAg genotype A	2
Ep#12	SSWAFAKYL	aa 153-161 of SHBsAg genotype A	1
Ep#13	IVSPFIPLL	aa 207-215 of SHBsAg genotype A	1
Ep#14	PYKMDIDPY	aa -3-6 of HBcAg, genotype A	2
Ep#15	SVRDLLDNASALYRE PSVRDLLDTASALYR	aa 26-40 of HBcAg,genotype D aa 25-39 of HBcAg,genotype A	2
Ep#16	TWVGNN	aa 70-75 of HBcAg genotype A	1
Ep#17	PRRRRSP	aa 165-171 of HBcAg genotype A	1

Interestingly, Ep#02, Ep#07, and Ep#09 were detected with the highest frequency (tables 16 & 17). In ongoing experiments with Prof. Dr. Stephan Urban, Department of Molecular Virology, University Clinic Heidelberg, IgG fractions will be isolated from human sera reacted with Ep#02, Ep#07, and Ep#09 in order to examine the reactivity with 4608_RPL. This will answer the question of whether antibodies from different sera recognize a similar set of random peptides.

Ep#2 recognized by five sera resides at the most N-terminal end of the HBV preS1 region. This region is important for HBV infectivity (Gripon, Le Seyec et al. 1995; Bruss, Hagelstein et al. 1996). Studies are underway to examine whether antibodies to Ep#2 neutralize HBV.

As seen in figures 33, 34, and 35, unfractionated sera created a background noise in the microarray, which is absent when purified mAbs are used (figures 16, 17, 19 and 20). As a negative control, sera from HBV-unvaccinated and HBV-negative individuals were screened with HBV microarray chips.

3.3.3 Synopsis and general conclusions

HBV contain three antigens, HBsAg, HBcAg and HBeAg, recognized by sera from HBV-infected individuals. The HBsAg comprises three antigenic regions: preS1, preS2 and SHBsAg. These three regions contain continuous epitopes (Alberti, Cavalletto et al. 1990; Folgori, Tafi et al. 1994; Germaschewski and Murray 1995; Germaschewski and Murray 1996; Park, Cho et al. 2000; Zhang, Wan et al. 2001; Hellstrom, Madalinski et al. 2009). These epitopes can be detected on the HBV microarray with sera from HBV-infected and/or -vaccinated individuals. Moreover, the microarrays detect continuous epitopes on the SHBsAg and HBcAg not identified earlier. The motif CKTCTT (aa 121-126 of SHBsAg, Ep#11) was only detected by sera from humans or mice inoculated with a yeast-derived HBV vaccine. The lack of reactivity of human sera with high HBs antibody titer on the HBV microarray is explained by the recognition of discontinuous epitopes. Screening the HBV serum samples from vaccinated individuals using the 4608_RPL failed due to both pre-immunized and post-immunized sera identifying the same pattern of peptides on the 4608_RPL. Affinity-purified antibodies from human sera are needed to identify motifs on 4608_RPL.

3.4 Reactivity of monkey sera to the HIVenv chips

Mapping targets of antibodies produced following vaccination is of great value for monitoring vaccination efficacy since targets may be identified as responsible for viral load reduction and protection. Monkey sera from three different immunization experiments conducted in the DPZ were screened with HIVenv chips (Dr. Christiane Stahl Hennig, Infection Models Unit, DPZ, provided us with these sera). In the first experiment (E1) (figure 36), DNA prime/MVA expressing HIV and SIV antigens were used (Stolte-Leeb, Bieler et al. 2008) and in the second (E2) (figure 37) DNA prime/Ad5 expressing also HIV and SIV antigens (Stahl-Hennig, Suh et al. 2007) were used. In the third experiment (E3) another adenovirus/SIV construct was employed. Immunized monkeys were challenged with SHIV 89.6P in E1 and E2 and with SIV mac239 in E3 and as a secondary challenge in E2.

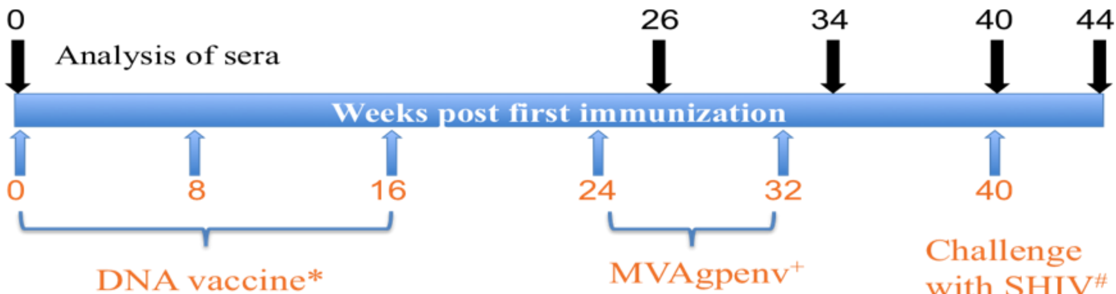


Figure 36: Vaccination regime and timing of sera collection in E1. * DNA vaccine: SIV gag/pol and HIV-1 env genes; + MVAgpenv: MVA expressing SIV gag/pol and HIV-1 89.6env; # SHIV: simian-human immunodeficiency virus 89.6P.

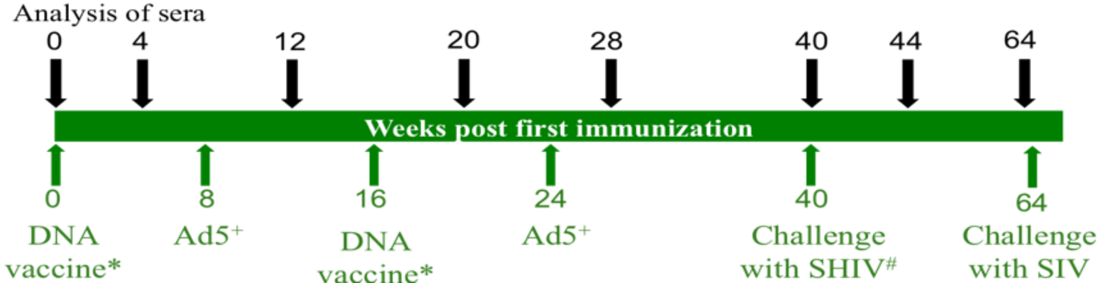


Figure 37: Vaccination regime and timing of sera collection in E2. * DNA vaccine: SIV Gag-Env, sPol, sVif-Nef and sTat-Vpx and HIV-1 Gag-Env and Tat-Vpu genes and other adjuvant gene; + Ad5: adenoviruses expressing SIV, HIV-1 and adjuvant genes (SIV Env, sPol, sVif-Nef and sTat-Vpx and HIV-1 Gag-Env and Tat-Vpu); # SHIV: simian-human immunodeficiency virus 89.6P.

Overall monkey sera from E1 (tables **S**13 & **S**14) and E2 (table **S**15) recognized 14 epitopes on the HIVenv chip (table 18 and figure 38). In these two experiments both HIV and SIV genes were included in the vaccine. Thus the question was whether the identified epitopes were specific for HIVenv and/or SIVenv. Monkeys of E3 were immunized only with adenovirus/SIV components. Monkey sera from E3 were screened with HIVenv chips. These sera did not recognise peptides on the HIVenv chip. Thus, epitopes identified with sera from E1 and E2 were due to HIV but not SIV immunization.

In E1, 11 epitopes were identified with a distribution between gp120 and gp41 of 8:3, while with E2 (8 epitopes), this ratio was 3:5 (table 18 and figure 38). These results show that the MVA used in E1 seems to facilitate preferentially the recognition of gp120 epitopes, while the adenovirus of E2 induces mainly antibodies to gp41 epitopes.

Five epitopes (HIV_Ep# 05, 08, 10, 11 and 12) (table 18 and dark blue triangle in figure 38) were identified with sera from both E1 and E2 as well as control groups (tables **S**12 & **S**16). Their presence was not correlated with reduced vial load.

Table 18: Epitopes mapped with monkey sera samples.

Epitope number	Spot number	Sequence	Site at HIVenv	Frequency			
				E1	E2	E3	control
HIV_Ep#01	23-26	PTDPNP	aa 76-81of gp120	03/08	-	-	-
HIV_Ep#02	34-36	EDIISLWDQ	aa106-114 of gp120	03/08	-	-	-
HIV_Ep#02A	52-54	NISTSIRGK	aa 160-168 of gp120	-	01/06	-	-
HIV_Ep#03	68-70	VSFEPIPIH	aa 208-216 of gp120	01/08	-	-	-
HIV_Ep#04	85-88	NGSLAE	aa 262-267 of gp 120	03/08	-	-	-
HIV_Ep#05	101-104	QRGPGR	aa 310-315 of gp 120	04/08	06/06	-	07/12
HIV_Ep#06	142-144	VGKAMYAPP	aa 450-458 of gp120	02/08	-	-	-
HIV_Ep#07	154-156	EIFRPGGGD	aa 466-474 of gp120	08/08	-	-	-
HIV_Ep#08	166-168	KRRVVQREK	aa 502-510 of gp120	06/08	05/06	-	08/12
HIV_Ep#09	178-180	TVQARQLLS	aa 538-546 of gp41	-	01/06	-	-
HIV_Ep#10	198-201	KLICTT	aa 601-606 of gp41	08/08	04/06	-	11/12
HIV_Ep#11	235-238 238-240	YSPLSF QTHLPTPRG	aa 712-717 of gp41 aa 718-726 of gp41	07/08	05/06	-	08/12
HIV_Ep#12	241-245	IEE	aa 733-735 gp41	08/08	06/06	-	08/12
HIV_Ep#13	280-282	HIPRRIRQGLE	aa 842-852 of gp41	-	03/06	-	-

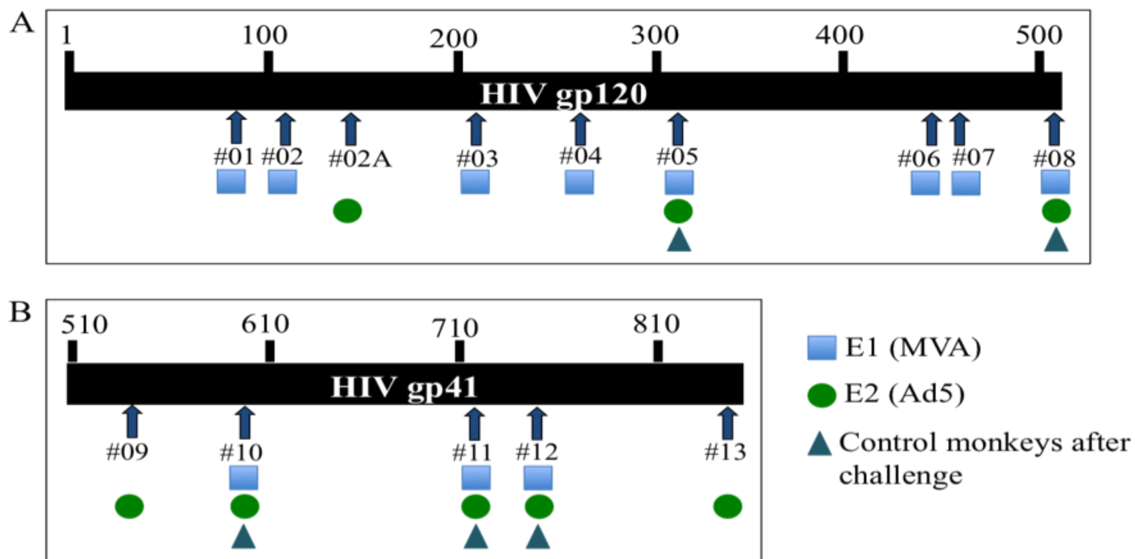


Figure 38: Locations of the 14 epitopes identified with monkey sera from E1, and E2 on gp120 (A) and gp41 (B).

Three monkeys in E2 recognized a unique epitope (HIV_Ep# 13) (figure 39). Two out of three animals had, to some extent, a lower peak of viremia after challenge with SHIV 89.6P (monkeys ID 11985, and 11986 in figure 40). Perhaps recognition of this epitope mediates some protection. HIV_Ep#13 is located at the C-terminal cytoplasmic tail of HIV gp41. This region has been identified earlier as a T-cell epitope (Frahm, Korber et al. 2004; Plana, Garcia et al. 2004). This is the first description of this B-cell epitope. Interestingly, the removal of this part of the HIV gp41 prevents the conformational change required for membrane fusion during viral entry (Costin, Rausch et al. 2007; Jiang and Aiken 2007).

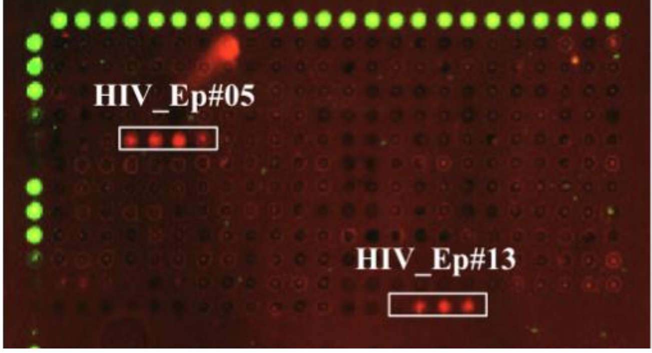


Figure 39: Reactive-peptides of one monkey serum from E2 on HIVenv chip.

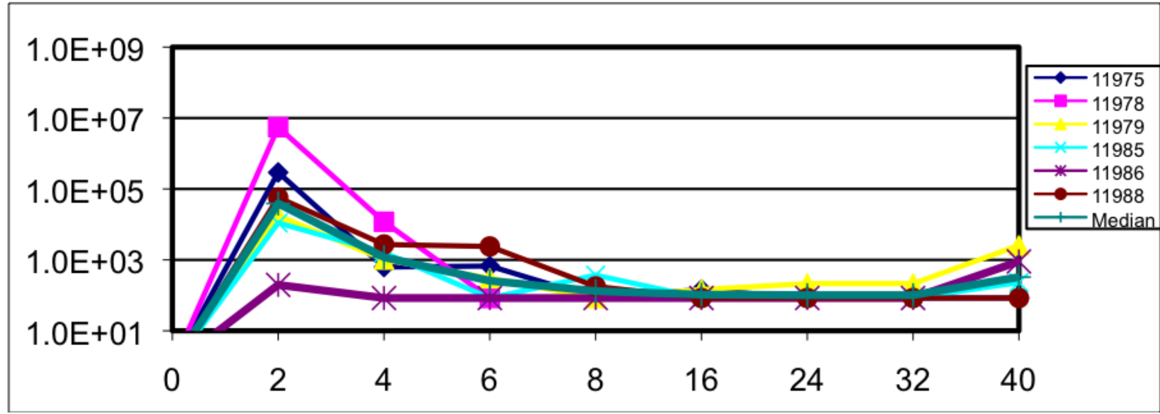


Figure 40: Viral loads in E2-vaccinated monkeys after rectal challenge with SHIV89.6P. RNA viral loads are expressed as RNA equivalents per milliliter of plasma of each animal for individual time points. The geometric mean of viral RNA copy numbers of each group at each time point is shown ± SD (unpublished data, provided by Dr. Christiane Stahl Hennig, Infection Models Unit, DPZ).

In conclusion, 14 epitopes were mapped using monkey sera from two immunization experiments conducted in DPZ. 13/14 has been mapped before (http://www.hiv.lanl.gov/). In this study, HIV_Ep#13 was identified for the first time as a B cell epitope. None of the immunized monkeys in these experiments is fully protected. Testing sera from fully protected vaccinees or individuals clear of the infection will give us more information about the correlation between antibody epitope and protection. Many researchers in the HIV vaccine field believed that an effective HIV vaccine must induce both broadly neutralizing antibodies and strong cell-mediated response (Baum 2010; Benmira, Bhattacharya et al. 2010). A recent study published in *Science* showed that a human mAb neutralizes over 90% of circulating HIV-1 isolates (Wu, Yang et al. 2010). Mapping targets of the broadly neutralizing antibody may help to design a vaccine able to produce such a response.

3.5 Cooperations

3.5.1 Neutralizing epitopes in the preS1 attachment site of hepatitis B virus are partially masked by N-terminal myristoylation

The preS1-domain of LHBsAg responsible for the attachment of HBV to the host cell via its N-terminal-myristoylated aa sequence 2-48 (Gripon, Le Seyec et al. 1995; Bruss, Hagelstein et al. 1996; Glebe, Urban et al. 2005; Glebe and Urban 2007). The neutralizing power of antibodies produced against this region is not known. To characterize this, the entire preS1 domains, C- and N-terminal preS1 stretches were inserted into the most immunogenic region of HBcAg to produce nine subviral particles (figure 41). BALB/c mice were immunized with these particles and the binding properties, and the neutralization potential of resulting antibodies were tested *in vitro* (figure 42) (Bremer, Sominskaya et al. 2010). The antibodies directed to the C-terminus of preS1 did not neutralize HBV infection *in vitro*, whereas, antibodies elicited to N-terminal of preS1 (aa 2-48) strongly neutralized the infection. Interestingly, antibodies against the very N-terminal part of preS1 (aa 1-21) were not able to neutralize although this sequence contains the most conserved part essential for HBV attachment to the cells.

To test whether the immunized mice produced antibodies against the inoculated construct, their sera were screened with the HBV microarray (figure 43). As expected, the sera identified the corresponding peptides (table 19). Sera against the preS1 N-terminus reacted mainly with peptides covering aa 1-33. Surprisingly, antibodies against preS1-core constructs that contained the full aa 2-48 sequence of preS1 reacted also with peptide aa 33-48, indicating a different conformation for these constructs in comparison to those without the aminoterminal preS1 aa 2-8. However, there was a unexpected low reaction of the antibodies against preS1 (aa 20-48) in the first three spots that might be explained by sequence homologies between spots number 131-333 and 136-139 (TxNP). Interestingly, an antiserum to preS1 (aa 1-21) recognized spots 1-7, which include preS1 aa 1-33, showing again, that the nonmyristoylated N-terminus of preS1 is very well recognized by the antibodies. Unfortunately, this antiserum did not prevent the HBV infection *in vitro* (figure 42). The N-terminal myristic acid presumably masks the most conserved part of the preS1 essential for viral attachment, thereby protecting against neutralizing antibodies, whereas antibodies to neighbouring sequences neutralize very well. This was a joint work with Prof. Dr. Wolfram Gerlich, University of Giessen, and

has been accepted for publication by the Journal of Hepatology (manuscript on the supplementary CD).

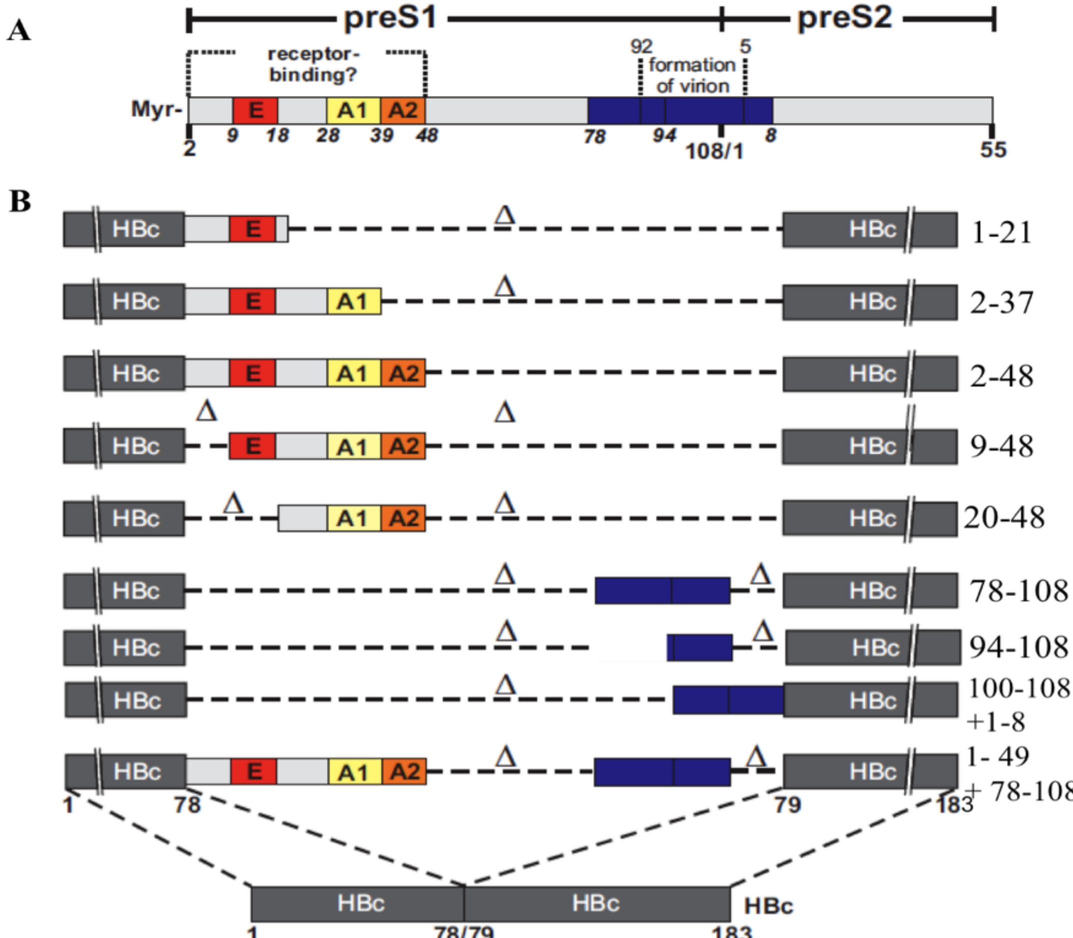


Figure 41: HBV preS-domain and peptide sequences inserted into HBc particles. (A) Structure of HB preS1 and preS2 domains. (B) PreS1 and preS2 short peptides inserted within the major immunogenic region of the HBcAg (Corinna M. Bremer, University of Giessen, personal communication).

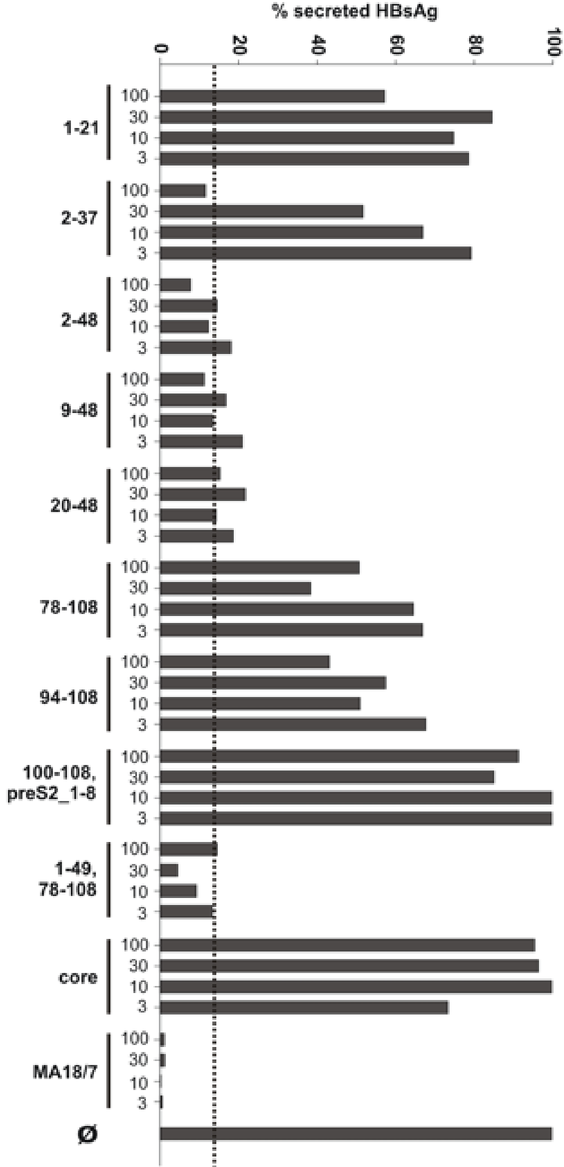


Figure 42: Neutralization of HBV-infection with the murine sera against the subviral particles. Highly purified HBV from chronic carriers was preincubated with the sera. Then, the mixture was incubated with primary tupaia hepatocytes. The neutralization power of sera was determined by the amount of HBsAg produced. The dotted line indicates the detection limit for HBsAg (Bremer CM et. al., accepted Journal of Hepatology).

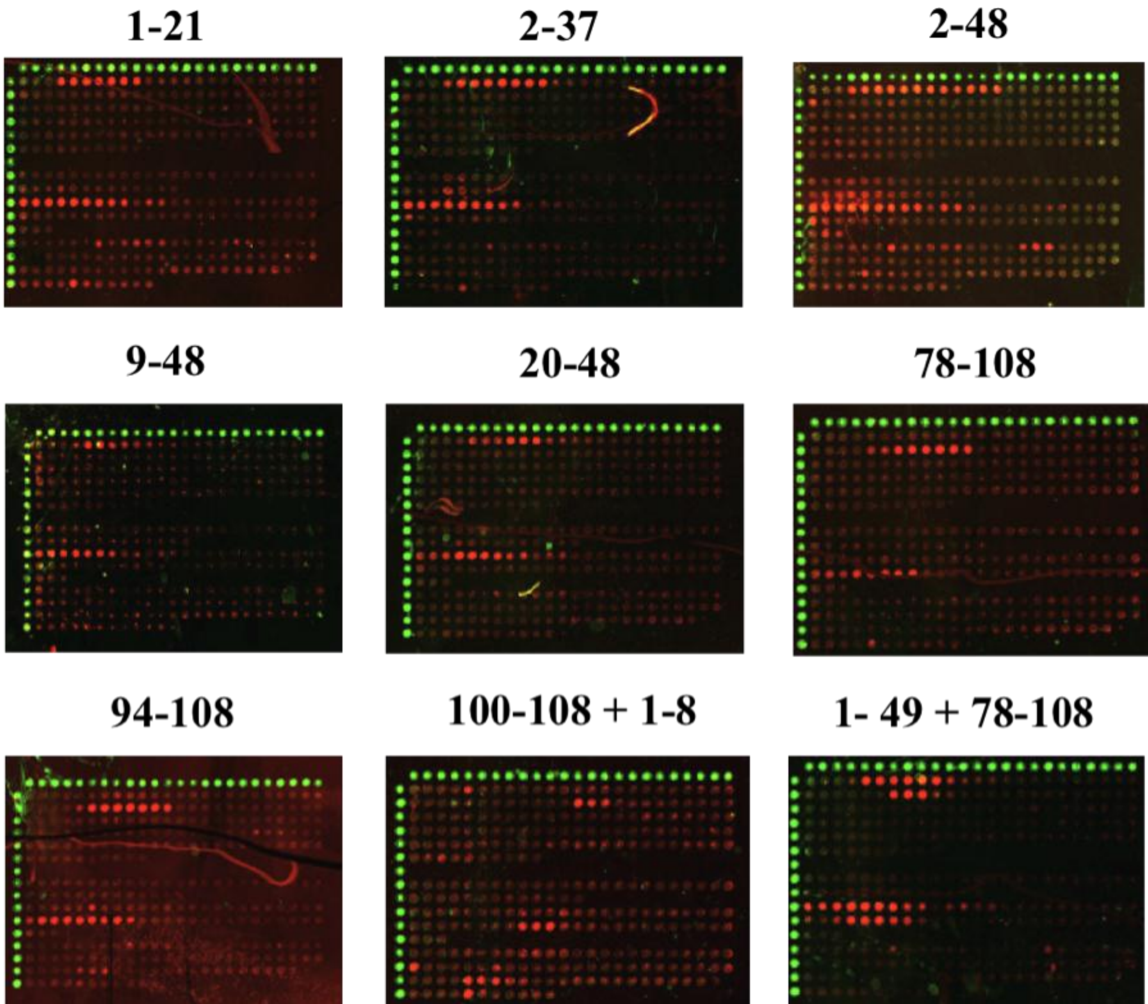


Figure 43: Reactivity of murine sera on the HBV microarray. The numbers of the reactive spots of each serum are given above each array. Sera identified sequence of both genotypes A and D. Many sera detected HBcAg sequences (1-21, 2-37, 2-48, 78-108, 94-108, 100-108+1-8, and 1-49+78-108).

Table 19: Sequence and position of the epitopes recognized by murine sera. Only the reactivity against preS1 and preS2 of HBV genotype D is shown.

Spot No.	Sequence	Position		1-21	2-37	2-48	9-48	20-48	78-108	94-108	100-108 +1-8	1-49 + 78-108	HBcAg
168	MGQNLSTSNPLGFFP	1	15										
169	NLSTSNPLGFFPDHQ	4	18										
170	TSNPLGFFPDHQLDP	7	21										
171	PLGFFPDHQLDPAFR	10	24										
172	FFPDHQLDPAFRANT	13	27										
173	DHQLDPAFRANTANP	16	30										
174	LDPAFRANTANPDWD	19	33										
175	AFRANTANPDWDFNP	22	36										
176	ANTANPDWDFNPNKD	25	39										
177	ANPDWDFNPNKDTWP	28	42										
178	DWDFNPNKDTWPDAN	31	45										
179	FNPNKDTWPDANKVG	34	48										
192	ILETLPANPPPASTN	73	87										
193	TLPANPPPASTNRQS	76	90										
194	ANPPPASTNRQSGRQ	79	93										
195	PPASTNRQSGRQPTP	82	96										
196	STNRQSGRQPTPLSP	85	99										
197	RQSGRQPTPLSPPLR	88	102										
198	GRQPTPLSPPLRNTH	91	105										
199	PTPLSPPLRNTHPQA	94	108										
200	LSPPLRNTHPQAMQW	97	3										
201	PLRNTHPQAMQWNST	100	6										
202	NTHPQAMQWNSTTFH	103	9										

red, strong responders; yellow, weak responders; blue, preS1 sequence; green, preS2 sequence.

3.5.2 Performance of polyclonal sera against preS1 and preS2 domains on the HBV microarray

α-Myrcludex B (myristoylated aa 2-48 of HBV preS1 domain) and recombinant hexa-histidine-preS1+preS2 fusion protein produced in E.coli were used to immunize two rabbits, AMBA and H863A, respectively (this experiment was performed in Prof. Dr. Stephan Urban's laboratory). Both immunogens were derived from the genotype D sequence. Sera from AMBA and H863A were screened with HBV microarray. As expected, AMBA reacted with aa 2-48 as this antigen was used for immunization (figure 44, table 20). Interestingly, H863A identified all preS1 and preS2 sequences on the HBV microarray, except spots 186-189, 199-205, and 213-218 (table 20). Thus these latter regions are not immunogenic. Because of the sequence difference between genotype A and D, H863A reacted with more peptides of genotype D than of genotype A. The

unexpected reactivity of AMBA and H863A sera with one peptide of HBcAg was detected. Perhaps these rabbits were exposed to other pathogens with HBV homologous sequences.

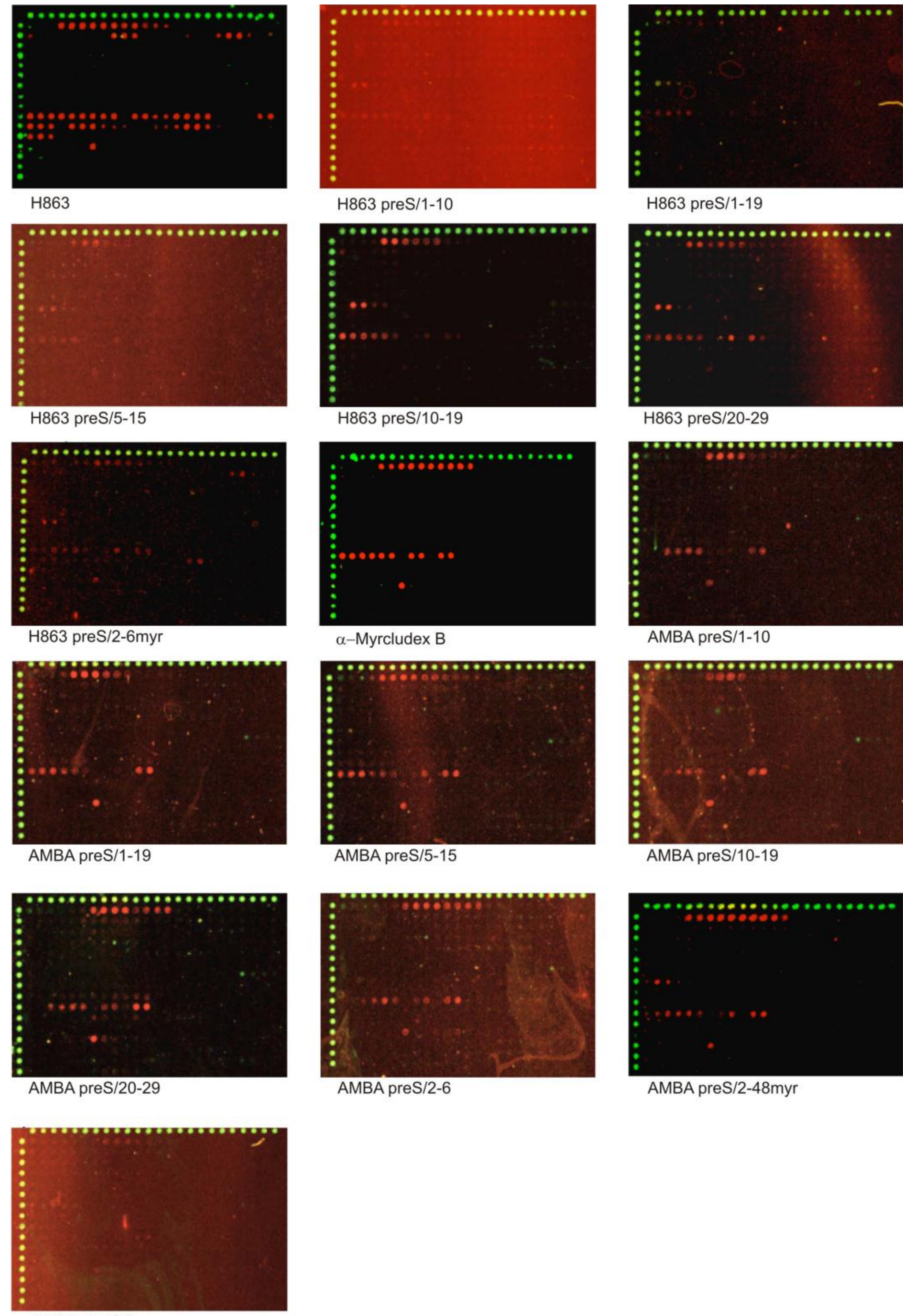


Figure 44: Reactivity of rabbit sera AMBA and H863A with the HBV microarray. Under each array the antibody fractions eluted from affinity column loaded with the serum indicated are shown.

Table 20: Sequence and position of the epitopes recognized by AMBA and H863A rabbit sera and their localization on the HBV microarray.

					H863A							AMBA								
Spot	Antigen	aa sequence	Gt A	Gt D	H863	preS/1-10	preS/1-19	preS/5-15	preS/10-19	preS/20-29	preS/2-6myr	AMBA	preS/1-10	preS/1-19	preS/5-15	preS/10-19	preS/20-29	preS/2-6myr	preS/2-48	myrGC
4	preS1 (A)	KGMGTNLSVPNPLGF	-2-13																	
5		GTNLSVPNPLGFFPD	2-16																	
6		LSVPNPLGFFPDHQL	5-19																	
7		PNPLGFFPDHQLDPA	8-22																	
8		LGFFPDHQLDPAFGA	11-25																	
9		FPDHQLDPAFGANSN	14-28																	
10		HQLDPAFGANSNNPD	17-31																	
11		DPAFGANSNNPDWDF	20-34																	
12		FGANSNNPDWDFNPI	23-37																	
13		NSNNPDWDFNPIKDH	26-40																	
14		NPDWDFNPIKDHWPA	29-43																	
43	preS2 (A)	FHQALQDPKVRGLYF	8-22									-	-	-	-	-	-	-	-	
44		ALQDPKVRGLYFPAG	11-25									-	-	-	-	-	-	-	-	
45		DPKVRGLYFPAGGSS	14-28									-	-	-	-	-	-	-	-	
46		VRGLYFPAGGSSSGT	17-31									-	-	-	-	-	-	-	-	
168	preS1 (D)	MGQNLSTSNPLGFFP		1-15																
169		NLSTSNPLGFFPDHQ		4-18																
170		TSNPLGFFPDHQLDP		7-21																
171		PLGFFPDHQLDPAFR		10-24																
172		FFPDHQLDPAFRANT		13-27																
173		DHQLDPAFRANTANP		16-30																
174		LDPAFRANTANPDWD		19-33																
175		AFRANTANPDWDFNP		22-36																
176		ANTANPDWDFNPNKD		25-39																
177		ANPDWDFNPNKDTWP		28-42	-															
178		DWDFNPNKDTWPDAN		31-45																
179		FNPNKDTWPDANKVG		34-48																
180		NKDTWPDANKVGAGA		37-51		-	-	-	-	-		-							-	
181		TWPDANKVGAGAFGL		40-54		-	-	-	-	-		-	-	-	-	-	-	-	-	
182		DANKVGAGAFGLGFT		42-57		-	-	-	-	-		-	-	-	-	-	-	-	-	
183		KVGAGAFGLGFTPPH		46-60		-	-	-	-	-		-	-	-	-	-	-	-	-	
184		AGAFGLGFTPPHGGL		49-63		-	-	-	-	-		-	-	-	-	-	-	-	-	
185		FGLGFTPPHGGLLGW		52-66		-	-	-	-	-		-	-	-	-	-	-	-	-	
190		SPQAQGILETLPANP		67-81		-	-	-	-	-		-	-	-	-	-	-	-	-	
191		AQGILETLPANPPPA		70-84		-	-	-	-	-		-	-	-	-	-	-	-	-	
192		ILETLPANPPPASTN		73-87		-	-	-	-	-		-	-	-	-	-	-	-	-	
193		TLPANPPPASTNRQS		76-90		-	-	-	-	-		-	-	-	-	-	-	-	-	
194		ANPPPASTNRQSGRQ		79-93		-	-	-	-	-		-	-	-	-	-	-	-	-	
196		STNRQSGRQPTPLSP		85-99		-	-	-	-	-		-	-	-	-	-	-	-	-	
197		RQSGRQPTPLSPPLR		88-102		-	-	-	-	-		-	-	-	-	-	-	-	-	
198		GRQPTPLSPPLRNTH		91-105		-	-	-	-	-		-	-	-	-	-	-	-	-	
206	preS2 (D)	TFHQTLQDPRVRGLY		7-21								-	-	-	-	-	-	-	-	
207		QTLQDPRVRGLYFPA		10-24								-	-	-	-	-			-	
208		QDPRVRGLYFPAGGS		13-27								-	-	-	-	-	-	-	-	
209		RVRGLYFPAGGSSSG		16-30								-	-	-	-	-	-	-	-	
210		TTVSPISSIFSRIGD		19-33								-	-	-	-	-	-	-	-	
211		SPISSIFSRIGDPAL		22-36								-	-	-	-	-	-	-	-	
212		PISSIFSRIGDPALN		25-39								-	-	-	-	-	-	-	-	
215	HBcrAg	ASKLCLGWLWGMDID		19-33																
301	myr Peptide	Myr-GTNLSVPNPLGFFPD	2-16		-							-	-	-	-	-	-	-		
302		Myr-MGQNLSTSNPLGFFP		2-16	-							-	-	-	-	-	-	-		

dark blue, strong responder; light blue, weak responder; yellow, signals too weak

Antibodies of AMBA and H863A sera were fractionated using peptide-coupled sepharose columns (performed at Prof. Dr. Stephan Urban´s laboratory). Six preS1 peptides were used for fractionation, aa1-10, aa 1-19, aa 5-15, aa 10-19, aa 0-29, and myristoylated aa 2-6 (2-6 myr), in addition to myristoylated GC (myrGC) as control. The 6 antibody fractions of both sera were screened with the HBV microarray (figure 44, table 20). The eluted antibodies did not react as expected. Even the control myrGC-eluted antibodies reacted weakly with four peptides on the HBV microarray. Moreover, 2-6 myr-eluted antibodies detected peptides not included in respective sequence, probably due to a general stickiness of preS1 antibody to myristoylated peptides. The fractionation of these sera should be repeated. In conclusion, the HBV microarray was a powerful tool as a control for the quality of the fractionation experiment. This work was a part of the bachelor thesis of Angela Brand, University Hospital Heidelberg.

3.5.3 Identification of mimotopes of an HSV mAb, 2c using the 4608_RPL

The 2c mAb (provided by Prof. Dr. Michael Roggendorf, Institute of Virology, University Clinic, Essen) was generated by immunizing mice with HSV-1 strain 342. It is specific for glycoprotein B of HSV. It had been shown to mediate clearance of infection from the mucous membranes of mice and to prevent ganglionic infection. *In vito*, the 2c mAb inhibits the viral cell-to-cell spread as well as syncytium formation (Eis-Hubinger, Mohr et al. 1991; Eis-Hubinger, Schmidt et al. 1993). The target sequence of the 2c mAb is unknown and thought to be a discontinuous epitope. The 2c mAb was screened with the 4608_RPL. It recognized 32 peptides, 8 strongly and 24 weakly (figure 45, table 21). No common motif could be extracted. Currently, the mimotopes reacting with high affinity to the 2c mAb are used to immunize mice to obtain antibodies with respective properties.

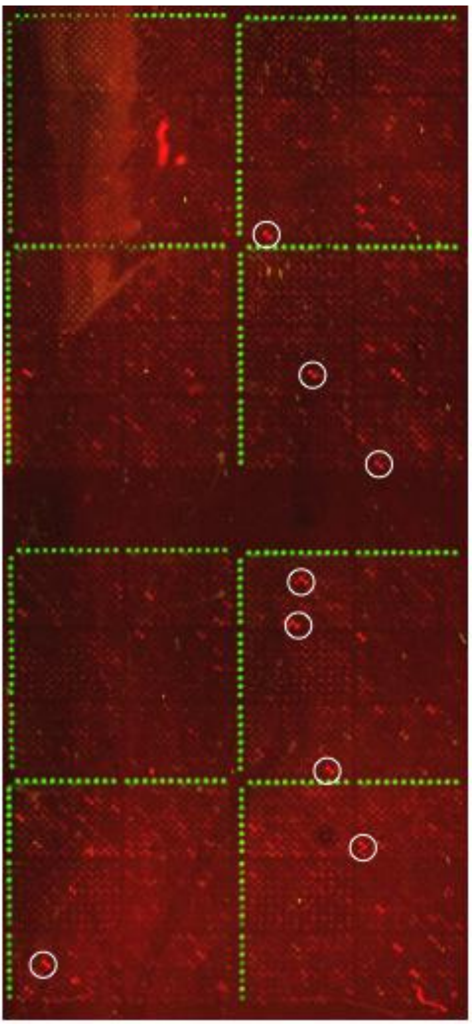


Figure 45: 2c mAb screened with 4608_RPL. The strong responders are circled in white.

Table 21: Peptides reactive with the 2c mAb.

No.	Spot No.	aa sequence	Reactivity	Remarks
1	Q11H3	LYAARSSDYFMCFDA	S	
2	Q19F8	MWSGHTHAQDFYFNA	S	
3	Q24H3	GGIFRGKPVYQEYHM	S	Reacts with secondary antibody
4	Q27C7	VRDNITKQKDYFELD	S	
5	Q27H6	YKWMDGYYHIAMHCF	S	
6	Q35H10	MTYCHVRYKDLNHEF	S	
7	Q40G1	TWMIHVKPDCSYYMG	S	
8	Q45D4	KASESNYYCIQFSTD	S	
9	Q12D5	EAEMFVLCCMSYYCT	W	
10	Q12E1	HAWFYCMYAGKFSTM	W	
11	Q12G2	WLMTNNDHYEWDDSY	W	
12	Q18F8	KKCNTWVNCHCYYDR	W	
13	Q14C11	TLCREIWCRYYYLRT	W	
14	Q22H3	YLIIHKMRIFARAFY	W	
15	Q20C2	DPRYRETQHHFHGDI	W	
16	Q20F5	RHKDYYCEMYNKEFH	W	
17	Q20H8	FPCCGMCNYDTYKQR	W	
18	Q24E1	KKAGVCMVSRMIRYY	W	
19	Q24D7	RHYIHYAAAERRFYN	W	Reacts with secondary antibody
20	Q21B10	QMDICVPNRAIGDDW	W	
21	Q26A12	VGINHYCQIVYTRYP	W	
22	Q26H11	AHHWNFYAQFRVRYY	W	
23	Q26E3	SRNSQCKGYMYFAVM	W	
24	Q27C6	WHYGKDHHYLFGAQG	W	
25	Q27H5	ERDFYHLCCLFYESQ	W	
26	Q32A1	NMYHEAWLYWEMNST	W	
27	Q35F2	ENTWNIWRRIYHVRW	W	
28	Q36A3	QSYFCEWKNRNTFDP	W	
29	Q36A9	SSDCASYFDVYAMSM	W	
30	Q39F4	RPYRSYHRRFMGWMD	W	Reacts with secondary antibody
31	Q48B5	CRYFQYYNNCLPPIM	W	
32	Q48F7	PSPFLFKSDQVYSKM	W	

S, strong responder; W, weak responder

4 Summary

Mapping targets of antibodies – in particular neutralizing ones – is of great value for monitoring vaccination efficacy as well as for development of new vaccines and reagents for diagnostics. To this end, several methods (*e.g.* phage display libraries, peptide-based ELISA) have been used. These, however, suffer from cumbersome handling and the need for large amounts of sera, often pooled from many patients with the effect of blinding the serological analysis to individual variation. As shown here, these disadvantages can be overcome by miniaturization and parallelization.

In this study, HBV and HIVenv microarray chips with overlapping oligopeptides encompassing the full amino acid sequences of different HBV and HIV polypeptides were produced via SPOT synthesis (Frank 1992) and printed onto glass slides. In addition, a random peptide library composed of 4608 15-mers was prepared. The chips were used for analyzing mAbs and sera from HBV-infected and -vaccinated individuals as well as HIV-vaccinated monkeys.

Targets of all mAbs used in this study directed to linear sequence were identified using HIVenv and HBV microarray chips. Interestingly, using HIVenv chips, antibodies with erroneously assigned epitopes have been identified in the EVA program catalogue. Mimotopes of antibodies recognizing different types of epitopes, continuous of 4 aa in length (MA18/7, and EVA3047 mAbs), discontinuous (C20/02 mAb), and those with modified residues (Q19/10 mAb), were identified using the 4608_RPL. However, the 4608_RPL could not identify sequences of mAbs with continuous epitopes of 6, 9, or 12 aa in length, possibly because of the constrain being too high. Nevertheless, mimics of targets of these mAbs were detected.

Seventeen HBV epitopes were identified with sera from HBV-infected and/or vaccinated individuals using HBV microarrays. Seven of them were detected for the first time. The CKTCTT motif (aa 121-126 of SHBsAg) was only reactive with sera of humans inoculated with a yeast-derived HBV vaccine, whereas, sera of humans vaccinated with mammalian-derived vaccine did not identify epitopes on the HBV microarray.

The HIVenv chips were used for screening monkey sera collected in three HIV/SIV vaccination experiments of the DPZ. In the E1, 8 rhesus monkeys were immunized with multigenic DNA prime/MVA boost vaccine, while in the second experiment E2, 6 rhesus (Stahl-Hennig, Suh et al. 2007) monkeys were immunized with DNA prime/adenovirus boost vaccine. In E3, another adenovirus/SIV construct was employed. Overall, 14 epitopes were identified. In E1, 11 epitopes were identified with a distribution between gp120 and gp41 of 8:3, while in E2 (8 epitopes), this ratio was 3:5. These results suggest that one and the same antigen, presented by two different vectors, can give rise to markedly different B-cell responses. Monkey sera from E3 did not recognise peptides on the HIVenv chip. Thus, epitopes identified with sera from E1 and E2 were due to HIV but not SIV immunization. Interestingly, three monkeys in E2 recognized a unique epitope (HIPRRIRQGLE, aa 842-852 of HIV gp41). This epitope was identified for the first time. In two-third of the monkeys, it had, to some extent, a lower peak of viremia after challenge with SHIV 89.6P. Perhaps recognition of this epitope mediates some protection. Nevertheless, more sera from vaccination experiments conducting the same regime must be tested.

Finally, the HBV and HIVenv microarray chips allowed for rapid (6 hours) and high-resolution mapping of B-cell continuous epitopes of HBV and HIV, respectively. The 4608_RPL is the starting point to develop a larger library that could serve as a universal tool to identify antibody targets (one chip serves all).

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6 Appendix (attached CD)

6.1 Microarray Excel program

6.1.1 HBV miniarray

6.1.2 HBV microarray

6.1.3 HIVenv microarray

6.1.4 4608_RPL

6.2 Supplementary tables

6.3 Supplementary figures

6.4 Published article

7 List of publications

Corinna M. Bremer, Irina Sominskaya, Dace Skrastina, Paul Pumpens, **Ahmed Abd El Wahed**, Ulrike Beutling, Ronald Frank, Hans-Joachim Fritz, Gerhard Hunsmann, Wolfram H. Gerlich and Dieter Glebe ´N-terminal myristoylation-dependent masking of neutralizing epitopes in the preS1 attachment site of hepatitis B virus." Journal of Hepatology, in print.

8 List of presentations at congresses and meetings

*** is the presented Author**

Beutling U., Städing K., Stradal T., Thiele S., **Abd El Wahed A.**, Fritz H.-J., Hunsmann G. and Frank R*. Cellulose-Bound Peptide Miniarrays for Systematic Studies of Peptide-Protein Interactions. The Proceedings of the 4th International Peptide Symposium in conjunction with the 7th Australian Peptide Conference and the 2nd Asia-Pacific International Peptide Symposium, 21-25 October, 2007, Cairns, Australia (**oral** presentation).

Abd El Wahed A. *, Beutling U., Frank R., Fritz H.-J., and Hunsmann G. Peptide miniarrays for the identification of hepatitis B virus epitopes. The XIV. International Congress of Virology, 10-15 August 2008, Istanbul, Turkey (**oral** presentation).

Ulrike Beutling*, **Ahmed Abd El Wahed,** Hans-Joachim Fritz, Gerhard Hunsmann, Kai Städing, Theresia Stradal and Ronald Frank. CELLULOSE-BOUND PEPTIDE MINI- AND MICROARRAYS FOR LARGE SCALE PEPTIDE SCREENING APPLICATIONS. The 9th German Peptide Symposium, March 11 - 14, 2009, Georg-August-University Goettingen, Germany (**poster** presentation).

Ahmed Abd El Wahed*, Ulrike Beutling, Ronald Frank, Gerhard Hunsmann and Hans-Joachim Fritz. Rapid and accurate detection of HIV epitopes using HIVenv scanning

chips prepared via SPOT synthesis. The 9th German Peptide Symposium, March 11 - 14, 2009, Georg-August-University Goettingen, Germany (**oral** presentation).

Corinna M. Bremer*, Irina Sominskaya, Dace Skrastina, Paul Pumpens, **Ahmed Abd El Wahed**, Hans-Joachim Fritz, Gerhard Hunsmann, Wolfram H. Gerlich and Dieter Glebe. Highly conserved and essential preS1 domain of hepatitis B virus (HBV) protected against binding of neutralising antibody by N-terminal myristoylation. 19. Annual Meeting of the German Society for Virology, 18-21. March 2009, Leipzig, Germany (**oral** presentation).

Ahmed Abd El Wahed*, Ulrike Beutling, Corinna M. Bremer, Aurelia Zvirbliene, Dieter Glebe, Wolfram H. Gerlich, Ronald Frank, Hans-Joachim Fritz and Gerhard Hunsmann. Identification of HBV epitopes via a rapid microchip assay reveals large differences in the immune response to yeast-derived HBsAg and natural or mammalian cell-derived HBsAg. 2009 International Meeting The Molecular Biology of Hepatitis B Viruses. August 30 - September 2, 2009 Tours, Loire Valley, France (**poster** presentation).

Corinna M. Bremer*, Irina Sominskaya, Dace Skrastina. Paul Pumpens, **Ahmed Abd El Wahed**, Ulrike Beutling, Ronald Frank, Hans-Joachim Fritz, Gerhard Hunsmann, Wolfram H. Gerlich and Dieter Glebe. N-terminal myristoylation protects the highly conserved and essential preS1 domain of hepatitis B virus (HBV) against binding of neutralizing antibodies. 2009 International Meeting The Molecular Biology of Hepatitis B Viruses. August 30 - September 2, 2009 Tours, Loire Valley, France (**poster** presentation).

Ahmed Abd El Wahed*, Ulrike Beutling, Ronald Frank, Gerhard Hunsmann and Hans-Joachim Fritz. Hepatitis B virus (HBV) and Human immunodeficiency virus (HIV) antibodies detected by peptide microarrays. Advances in Microarray Technology congress, 25-26 May 2010, Dublin, Ireland (**poster** presentation).

Ahmed Abd El Wahed*, Ulrike Beutling, Christiane Stahl-Hennig, Ronald Frank, Gerhard Hunsmann and Hans-Joachim Fritz. Peptide Microarray for Mapping HIVgp160 Epitopes after Vaccination of Rhesus Monkeys against HIV/SIV with Modified Vaccinia Ankara or Adenovirus Vectors. 4th Vaccine and ISV Annual Global Congress held in Vienna in 3-5 October 2010, Vienna, Austria (**oral** presentation).

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10 Curriculum Vitae

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